THE JOURNAL

OF

EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D.

VOLUME THIRTY-SECOND

WITH ONE HUNDRED PLATES AND SIXTY-ONE
FIGURES IN THE TEXT

NEW YORK
THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH
1920

WAVERLY PRESS
The Williams & Wilkins Company
Baltimore, U. S. A.

CONTENTS.

No. 1, July 1, 1920.

No. 2, August 1, 1920.

NO. 3, SEPTEMBER 1, 1920.

No. 4, October 1, 1920.

No. 5, November 1, 1920.

STUDIES ON EXPERIMENTAL PNEUMONIA.

VII. Treatment of Experimental Pneumococcus Type I Pneumonia in Monkeys with Type I Antipneumococcus Serum.

By RUSSELL L. CECIL, M.D., and FRANCIS G. BLAKE, M.D.

(*From the Bacteriological Laboratory of the Army Medical School, Washington.*)

(Received for publication, January 23, 1920.)

The treatment of Pneumococcus Type I pneumonia with Type I antipneumococcus serum has come into extensive use since the original studies in 1913 by Cole[1] and his coworkers. These investigators found that Pneumococcus Type I pneumonia, when not treated with serum exhibited a mortality rate of about 25 per cent. In a large series of serum-treated cases, however, they showed that the mortality rate could be reduced from this high figure to approximately 7 per cent. In spite of these convincing studies, Type I antipneumococcus serum has not come into such wide use as its efficacy would seem to justify. The reasons for this are several. Among them may be mentioned lack of familiarity with the subject, inexperience with the technique of administering serum, difficulty in obtaining a prompt determination of the type of pneumococcus, and finally a certain skepticism among many physicians as to the therapeutic value of serum treatment. Especially in the Army, in the various base hospitals where Type I antipneumococcus serum was used extensively, able clinicians were often heard to express doubt as to the usefulness of any kind of serum treatment in pneumonia.

The proper administration of Type I antipneumococcus serum in Pneumococcus Type I pneumonia has been followed, in the case of human beings, by excellent results, as many hospital records show. So far as known, however, no test of its value has ever been made in

[1] Cole, R., *J. Am. Med. Assn.*, 1913, lxi, 663; 1917, lxix, 505; *Arch. Int. Med.*, 1914, xiv, 56.

experimental pneumonia in animals when treated and untreated cases could be compared simultaneously. The studies of experimental pneumonia in monkeys which have been previously presented[2] show that pneumococcus pneumonia in monkeys differs in no essential respect either clinically or pathologically from pneumococcus pneumonia in man. The present study was undertaken, therefore, to determine by experiments on monkeys the therapeutic value of Type I antipneumococcus serum in Pneumococcus Type I pneumonia.

Method.

The Philippine *Macacus* (*Macacus syrichtus*) was used in all the experiments reported in this paper. Pneumococcus Type I pneumonia was induced in them, according to the method previously described, by the intratracheal injection of a highly virulent Pneumococcus Type I in amounts which experience had shown invariably produced a fatal pneumonia.

Type I antipneumococcus serum, prepared by the New York State Board of Health, was employed throughout. The serum, which was in no case administered until the animal had developed definite symptoms of pneumonia, was diluted in most instances with an equal quantity of normal salt solution, and slowly injected by means of a Luer syringe into the femoral vein. The dose employed was 10 cc. except in one experiment where doses of 20 cc. were given in order to determine the effect of large dosage. The dose of antipneumococcus serum recommended for man is 90 to 100 cc. As a Philippine *Macacus* weighs about one-sixteenth as much as man, the monkeys, in receiving only 10 cc., got a somewhat larger dose in proportion to their weight than a human being receives.

Effect of Type I Antipneumococcus Serum When Administered Early in the Disease.

Hospital patients suffering with Pneumococcus Type I pneumonia rarely receive their first injection of serum until 24 or 48 hours after the onset of the disease; in many cases even more time elapses before

[2] Blake, F. G., and Cecil, R. L., *J. Exp. Med.*, 1920, xxxi, 403, 445, 499. Cecil, R. L., and Blake, F. G., *J. Exp. Med.*, 1920, xxxi, 519, 657, 685.

serum treatment is instituted. In the experiment about to be reported serum treatment was started 24 hours after the pneumococci were injected into the trachea of the monkey in order to determine the effect of antipneumococcus serum when administered early in the course of the disease. Monkey 94, which received 0.1 cc. of Pneumococcus Type I, was treated with serum. Monkey 93, which received only 0.001 cc. of Pneumococcus Type I, served as a control (Text-fig. 1).

Experiment 1.—Monkey 94 (Text-fig. 1). *Macacus syrichtus*, male; weight 4,602 gm.

May 12, 1919. Well and active.

May 14. Well and active.

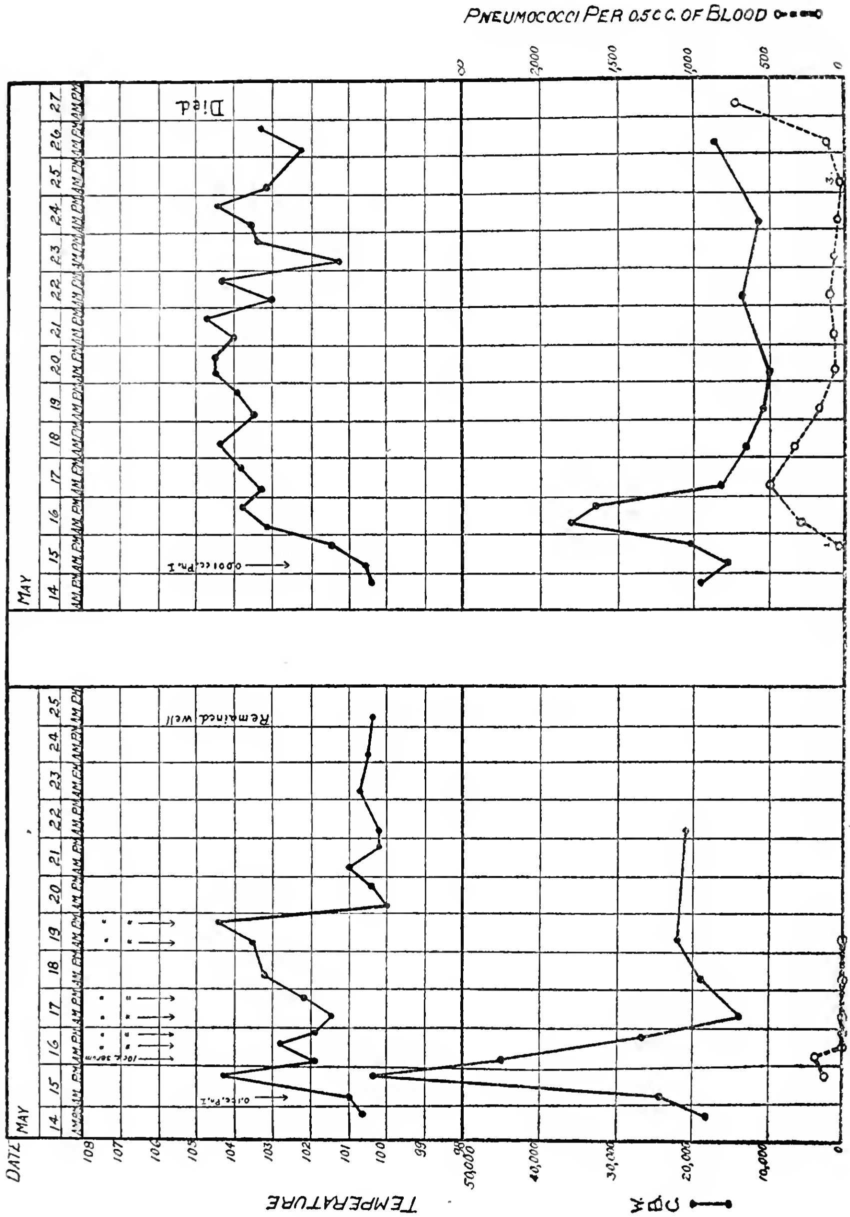
May 15. Well and active. 10.35 a.m. Intratracheal injection of 0.1 cc. of 18 hour broth culture of Pneumococcus Type I (in 1 cc. of broth). 4.10 p.m. Quiet. Respirations moderately accelerated. Blood culture: 0.5 cc. in broth, Pneumococcus Type I; 0.5 cc. in agar plate, 43 colonies of Pneumococcus Type I.

May 16, 9.55 a.m. Appears moderately sick; huddled up on perch. Breathing rapid and labored. Blood culture: 0.5 cc. in broth, Pneumococcus Type I; 0.5 cc. in agar plate, 103 colonies of Pneumococcus Type I. 10 a.m. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution. No reaction. 3.55 p.m. Quiet. Breathing moderately accelerated. Blood culture: 0.5 cc. in broth, no growth; 0.5 cc. in agar plate, no growth. 4 p.m. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution. No reaction. 9 p.m. Quiet but appears in good condition; breathing easily. Blood culture: 0.5 cc. in broth, no growth; 0.5 cc. in agar plate, no growth. 9.30 p.m. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution (about half of material escaped into surrounding tissue).

May 17. Appears in good condition; breathing easily; temperature normal. 10.05 a.m. Blood culture: 0.5 cc. in broth, no growth; 0.5 cc. in agar plate, no growth. 10.10 a.m. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution. No reaction. 4 p.m. Monkey appears well; breathing normally. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution. No reaction.

May 18. Appears in good condition; breathing easily; temperature, however, has gone up. 11 a.m. Blood culture: 0.5 cc. in broth, no growth; 0.5 cc. in agar plate, no growth.

May 19. Monkey appears in excellent condition; respiration quiet and slow; temperature still up, however. 11.45 a.m. Blood culture: 0.5 cc. in broth, no growth; 0.5 cc. in agar plate, no growth. 11.50 a.m. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution.



TEXT-FIG. 1, *a* and *b*. Experimental Pneumococcus Type I pneumonia treated with Type I antipneumococcus serum. (*a*) Monkey 94; treated with Type I antipneumococcus serum. (*b*) Monkey 93; untreated control.

No reaction. 4.30 p.m. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution.

May 20. Appears well and active. Temperature has fallen to normal by crisis.

May 21 to 26. Well and active.

May 28. Appears well and active. Slight rise in temperature during past 2 days may be due to serum disease though no other manifestations of the condition have appeared.

May 29. Well.

June 2. Continues well.

Monkey 93 (Text-fig. 1). *Macacus syrichtus*, male; weight 5,110 gm. Control for Monkey 94.

May 12, 1919. Well and active.

May 14. Well and active.

May 15. Well and active. 10.25 a.m. Intratracheal injection of 0.001 cc. of 18 hour broth culture of Pneumococcus Type I (in 1 cc. of broth). 4 p.m. Appears well. Blood culture: 0.5 cc. in broth, Pneumococcus Type I; 0.5 cc. in agar plate, 1 colony of Pneumococcus Type I.

May 16. Moderately sick; respirations increased. 10.10 a.m. Blood culture: 0.5 cc. in broth, Pneumococcus Type I; 0.5 cc. in agar plate, 337 colonies of Pneumococcus Type I.

May 17. Monkey sick; respirations rapid and labored; suggestive signs of involvement of right lower lobe. 10.20 a.m. Blood culture: 0.5 cc. in broth, Pneumococcus Type I; 0.5 cc. in agar plate, 500 colonies of Pneumococcus Type I.

May 18, 10.45 a.m. Condition the same; respirations rapid and labored. Blood culture: 0.5 cc. in broth, Pneumococcus Type I; 0.5 cc. in agar plate, 365 colonies of Pneumococcus Type I.

May 19. Sick; breathing rapidly; dullness and suppressed breathing over right lower lobe. 11.20 a.m. Blood culture: 0.5 cc. in broth, Pneumococcus Type I; 0.5 cc. in agar plate, 216 colonies of Pneumococcus Type I.

May 20. Appears sick; sitting huddled up; breathing rapidly. 11.35 a.m. Blood culture: 0.5 cc. in broth, Pneumococcus Type I; 0.5 cc. in agar plate, 24 colonies of Pneumococcus Type I.

May 21. Condition the same. 11.25 a.m. Blood culture: 0.5 cc. in broth, Pneumococcus Type I; 0.5 cc. in agar plate, 35 colonies of Pneumococcus Type I.

May 22. Sick; respirations rapid and labored. 4.15 p.m. Blood culture: 0.5 cc. in broth, Pneumococcus Type I; 0.5 cc. in agar plate, 73 colonies of Pneumococcus Type I.

May 23. Still sick; respirations rapid and grunting; marked dullness over entire right chest, and bronchial breathing. 10.55 a.m. Blood culture: 0.5 cc. in broth, Pneumococcus Type I; 0.5 cc. in agar plate, 66 colonies of Pneumococcus Type I.

May 24. Condition the same. 10.45 a.m. Blood culture: 0.5 cc. in broth, Pneumococcus Type I; 0.5 cc. in agar plate, 12 colonies of Pneumococcus Type I.

May 25. Still sick; respirations rapid and grunting. 10.30 a.m. Blood culture: 0.5 cc. in broth, Pneumococcus Type I; 0.5 cc. in agar plate, 3 colonies of Pneumococcus Type I.

May 26. Seems better but still sick. 10.20 a.m. Blood culture: 0.5 cc. in broth, Pneumococcus Type I; 0.5 cc. in agar plate, 46 colonies of Pneumococcus Type I.

May 27. Appears very sick; lying on floor of cage most of time. 11.20 a.m. Blood culture: 0.5 cc. in broth, Pneumococcus Type I; 0.5 cc. in agar plate, 750 colonies of Pneumococcus Type I. 3.15 p.m. Died.

Autopsy.—May 27, 4 p.m., immediately after death.

Anatomical Diagnosis.—Experimental lobar pneumonia (Pneumococcus Type I), right upper, middle, and lower lobes; acute fibrinous pleuritis, right side; Pneumococcus Type I septicemia; hypertrophy and dilatation of the heart; cloudy swelling of viscera.

Subject.—Large male *Macacus.* Body is still warm. Abdominal organs normally disposed; liver and kidneys show cloudy swelling. Pericardial and left pleural cavities are normal. There are fresh fibrinous adhesions throughout the right pleural cavity.

Heart.—Markedly enlarged; the cavities on both the right and left sides show considerable dilatation. The heart muscle and valves appear normal. *Left lung.*—Somewhat collapsed, pale, and crepitant throughout. *Right lung.*—Entire lung shows a massive consolidation. It is considerably larger and heavier than the left lung, and its surface is covered with dark red fibrinous tags. Cut surface of right lung shows typical gray hepatization. *Trachea and bronchi.*—Filled with frothy mucus. *Bronchial lymph glands.*—Enlarged.

Microscopic Examination.—*Right lower lobe.*—A considerable part of the section is necrotic. Inside the necrotic areas there are irregular conglomerations of polymorphonuclear leucocytes, many of them undergoing necrosis and disintegration. In the portion of the section which is still preserved, the tissue shows the typical picture of resolution associated with early organization. There are swelling and desquamation of the alveolar epithelium with plugs of hyaline material in many of the alveoli. A few poorly staining pneumococci are found in the section. *Bronchial lymph glands.*—Sinuses contain large macrophage cells and a moderate number of lymphoid cells.

Autopsy Cultures.—Heart's blood, Pneumococcus Type I; right bronchus, abundant colonies of Pneumococcus Type I; right lower lobe, few colonies of Pneumococcus Type I.

These two protocols indicate that both monkeys promptly developed pneumonia following intratracheal inoculation with Pneumococcus Type I. In Text-fig. 1 it will be seen that both monkeys

presented sharp temperature and leucocyte reactions and both developed positive blood cultures. The institution of serum treatment, however, in Monkey 94 had a marked effect on the temperature which dropped almost immediately to normal. Moreover, the blood culture which had shown 103 colonies per 0.5 cc. of blood promptly became sterile following the first injection of 10 cc. of serum. The importance of keeping up serum treatment even after the temperature has dropped is well shown in this experiment. When the temperature had been practically normal for 2 days, it was decided to discontinue the serum treatment. On the following morning, however, the temperature was at 103.2°F., and on the next day it rose to 104.5°. Serum treatment was again started and the temperature dropped by crisis from 104.5° to 100° and remained normal thereafter.

There can be no reasonable doubt that in this case serum treatment greatly shortened the course of the disease and saved the monkey's life. It received 0.1 cc. of broth culture of Pneumococcus Type I, a dose which has, in our experience, been invariably fatal. The control monkey received only 0.001 cc. of broth culture and died on the 13th day of the disease. This experiment also shows that serum treatment when administered early promptly removes pneumococci from the blood, and shortens the course of the disease.

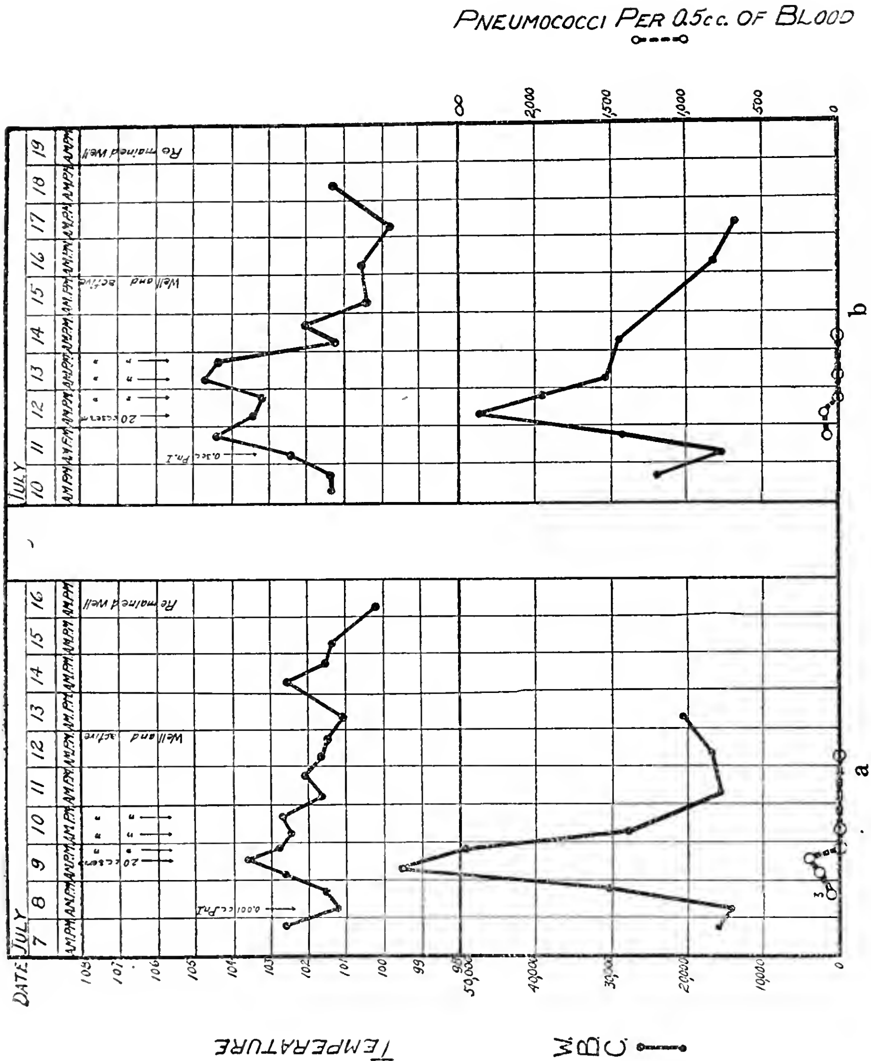
Effect of Large Doses of Serum Administered Early in the Disease.—The object of the following experiment was to produce an abortive form of pneumonia by frequent injections of large doses of serum early in the disease.

Experiment 2.—Monkey 45 (Text-fig. 2). *Macacus syrichtus*, male; weight 4,544 gm.

July 7, 1919. Well and active.

July 8. Well and active. 10 a.m. Intratracheal injection of 0.001 cc. of 18 hour broth culture of Pneumococcus Type I (in 1 cc. of broth). 3.50 p.m. Seems well. Blood culture: 0.5 cc. in broth, Pneumococcus Type I; 0.5 cc. in agar plate, 3 colonies of Pneumococcus Type I.

July 9, a.m. Having a severe chill; respirations moderately accelerated. Blood culture: 0.5 cc. in broth, Pneumococcus Type I; 0.5 cc. in agar plate, 37 colonies of Pneumococcus Type I. 3 p.m. Sitting huddled up with rapid grunting respiration; evidently in pain; marked hyperesthesia in left axilla. Blood culture: 0.5 cc. in broth, Pneumococcus Type I; 0.5 cc. in agar plate, 111



TEXT-FIG. 2, *a* and *b*. Abortive Pneumococcus Type I pneumonia, produced by early administration of large doses of Type I antipneumococcus serum. (*a*) Monkey 45. (*b*) Monkey 79.

colonies of Pneumococcus Type I. 3.05 p.m. Intravenous injection of 20 cc. of Antipneumococcus Serum Type I. 9 p.m. Appears better but still breathing rapidly. Blood culture: 0.5 cc. in broth, no growth; 0.5 cc. in agar plate, no growth. 9.05 p.m. Intravenous injection of 20 cc. of Antipneumococcus Serum Type I.

July 10. Quiet but otherwise appears well. Breathing easily. 10 a.m. Blood culture: 0.5 cc. in broth, no growth; 0.5 cc. in agar plate, no growth. 10.05 a.m. Intravenous injection of 20 cc. of Antipneumococcus Serum Type I. 3.45 p.m. Quiet but otherwise appears well. Intravenous injection of 20 cc. of Antipneumococcus Serum Type I.

July 11. Well and active; breathing easily; appears to have completely recovered.

July 12. Appears well and active. 10.20 a.m. Blood culture: 0.5 cc. in broth, no growth; 0.5 cc. in agar plate, no growth.

July 13 to 16. Well and active.

Monkey 79 (Text-fig. 2). *Macacus syrichtus*, male; weight 6,425 gm.

July 10, 1919. Well and active.

July 11. Well and active. 10.10 a.m. Intratracheal injection of 0.3 cc. of 18 hour broth culture of Pneumococcus Type I. 4 p.m. Quiet. Blood culture: 0.5 cc. in broth, Pneumococcus Type I; 0.5 cc. in agar plate, 11 colonies of Pneumococcus Type I.

July 12. Appears moderately sick; breathing rapidly. 9.55 a.m. Blood culture: 0.5 cc. in broth, Pneumococcus Type I; 0.5 cc. in agar plate, 15 colonies of Pneumococcus Type I. 10 a.m. Intravenous injection of 20 cc. of Antipneumococcus Serum Type I. Vomited 5 minutes later. 3.45 p.m. Appears better; breathing quieter. Blood culture: 0.5 cc. in broth, no growth; 0.5 cc. in agar plate, no growth. 4 p.m. Intravenous injection of 20 cc. of Antipneumococcus Serum Type I. No reaction.

July 13. Quiet; refuses food; breathing moderately accelerated. 10.30 a.m. Blood culture: 0.5 cc. in broth, no growth; 0.5 cc. in agar plate, no growth. 10.35 a.m. Intravenous injection of 20 cc. of Antipneumococcus Serum Type I. 6 p.m. Intravenous injection of 20 cc. of Antipneumococcus Serum Type I.

July 14. Temperature has fallen to normal by crisis. Appears well and active.

July 15 to 18. Well and active.

Both the monkeys in this experiment presented symptoms of pneumonia following the intratracheal injection of Pneumococcus Type I. In both there were sharp temperature and leucocyte reactions and both monkeys promptly developed positive blood cultures. In Monkey 45 serum treatment was started 29 hours after intratracheal injection. Reference to Text-fig. 2 shows that the object of the experiment was attained; namely, an abortive pneumonia. The tempera-

ture immediately returned to normal, the blood culture became sterile 6 hours after the injection of serum, and 4 days after intratracheal injection the monkey was entirely well and remained so. Monkey 79 received a much larger dose of culture (0.3 cc.) than Monkey 45 received, and for that reason the infection was not so promptly overcome as in Monkey 45. Even here, however, four large doses of serum were sufficient to bring the temperature down to normal, where it remained permanently. The blood culture followed the usual course in serum-treated animals and was sterile 6 hours after serum treatment was started. This experiment shows that by doubling the size of the dose serum treatment was made even more rapidly efficacious than in Experiment 1. An abortive type of pneumonia was produced in which the acute symptoms lasted only 24 to 48 hours.

Effect of Type I Antipneumococcus Serum When Administered Late in the Disease.

In the two preceding experiments the value of early serum treatment has been demonstrated. In the following experiments it was desired to determine the effect of serum treatment when initiated late in the disease. In the first monkey (No. 99) serum treatment was not started until the 3rd day of the disease, in the second monkey (No. 113) not until the 5th day. Both monkeys received doses of culture (0.01 cc.) which without serum treatment had invariably proved fatal. The control (Monkey 112) received the same dose of culture intratracheally and was treated with normal horse serum (Text-fig. 3).

Experiment 3.—Monkey 99 (Text-fig. 3). *Macacus syrichtus,* male; weight 6,020 gm.

June 4, 1919. Well and active.

June 5. Well and active. 10.30 a.m. Intratracheal injection of 0.01 cc. of 18 hour broth culture of Pneumococcus Type I (in 1 cc. of broth). 4.30 p.m. Blood culture: 0.5 cc. in broth, Pneumococcus Type I; 0.5 cc. in agar plate, 26 colonies of Pneumococcus Type I.

June 6, 10.30 a.m. Moderately sick; respirations rapid. Blood culture: 0.5 cc. in broth, Pneumococcus Type I; 0.5 cc. in agar plate, 650 colonies of Pneumococcus Type I.

June 7. Sick; respirations rapid and labored. 9.55 a.m. Blood culture: 0.5 cc. in broth, Pneumococcus Type I; 0.5 cc. in agar plate, 800 colonies of Pneumococcus Type I. 10 a.m. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution. 3.55 p.m. No definite improvement. Blood culture: 0.5 cc. in broth, no growth; 0.5 cc. in agar plate, no growth. 4 p.m. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution. 9.15 p.m. Still sick but not so toxic. Blood culture: 0.5 cc. in broth, no growth; 0.5 cc. in agar plate, no growth. 9.20 p.m. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution.

June 8. Temperature still elevated but monkey appears better; respirations moderately accelerated. 10.30 a.m. Blood culture: 0.5 cc. in broth, no growth; 0.5 cc. in agar plate, no growth. 10.35 a.m. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution. 4.30 p.m. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution.

June 9. Marked improvement; seems fairly well; respirations only moderately accelerated; temperature still elevated, however. 10.15 a.m. Blood culture: 0.5 cc. in broth, no growth; 0.5 cc. in agar plate, no growth. 10.20 a.m. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution. 4 p.m. Condition the same. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution. 9 p.m. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution.

June 10. Appears in good condition but moderately sick. 10 a.m. Blood culture: 0.5 cc. in broth, no growth; 0.5 cc. in agar plate, no growth. 10.15 a.m. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution. 4.15 p.m. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution. 9.15 p.m. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution.

June 11. Improving; more active; respirations still accelerated; dullness over right lower lobe. 10 a.m. Blood culture: 0.5 cc. in broth, no growth; 0.5 cc. in agar plate, no growth. 10.05 a.m. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution. 4.05 p.m. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution.

June 12. Shows marked improvement; respirations easy; temperature falling. 10 a.m. Blood culture: 0.5 cc. in broth, no growth; 0.5 cc. in agar plate, no growth. 10.05 a.m. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution. 4 p.m. Temperature normal; condition excellent. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution.

June 13, 16, 18, 24. Continues well.

Monkey 113 (Text-fig. 3). *Macacus syrichtus*, male; weight 5,285 gm.

June 4, 1919. Well and active.

June 5. Well and active. 10.45 a.m. Intratracheal injection of 0.01 cc. of 18 hour broth culture of Pneumococcus Type I (in 1 cc. of broth). 4.45 p.m. Blood culture: 0.5 cc. in broth, no growth; 0.5 cc. in agar plate, 1 colony of Pneumococcus Type I.

June 6, 10.40 a.m. Moderately sick; respirations rapid. Blood culture: 0.5 cc. in broth, Pneumococcus Type I; 0.5 cc. in agar plate, 88 colonies of Pneumococcus Type I.

June 7. Sick; respirations rapid and labored. 10.30 a.m. Blood culture: 0.5 cc. in broth, Pneumococcus Type I; 0.5 cc. in agar plate, 400 colonies of Pneumococcus Type I.

June 8. Sick; respirations rapid and labored. 11 a.m. Blood culture: 0.5 cc. in broth, Pneumococcus Type I; 0.5 cc. in agar plate, 69 colonies of Pneumococcus Type I.

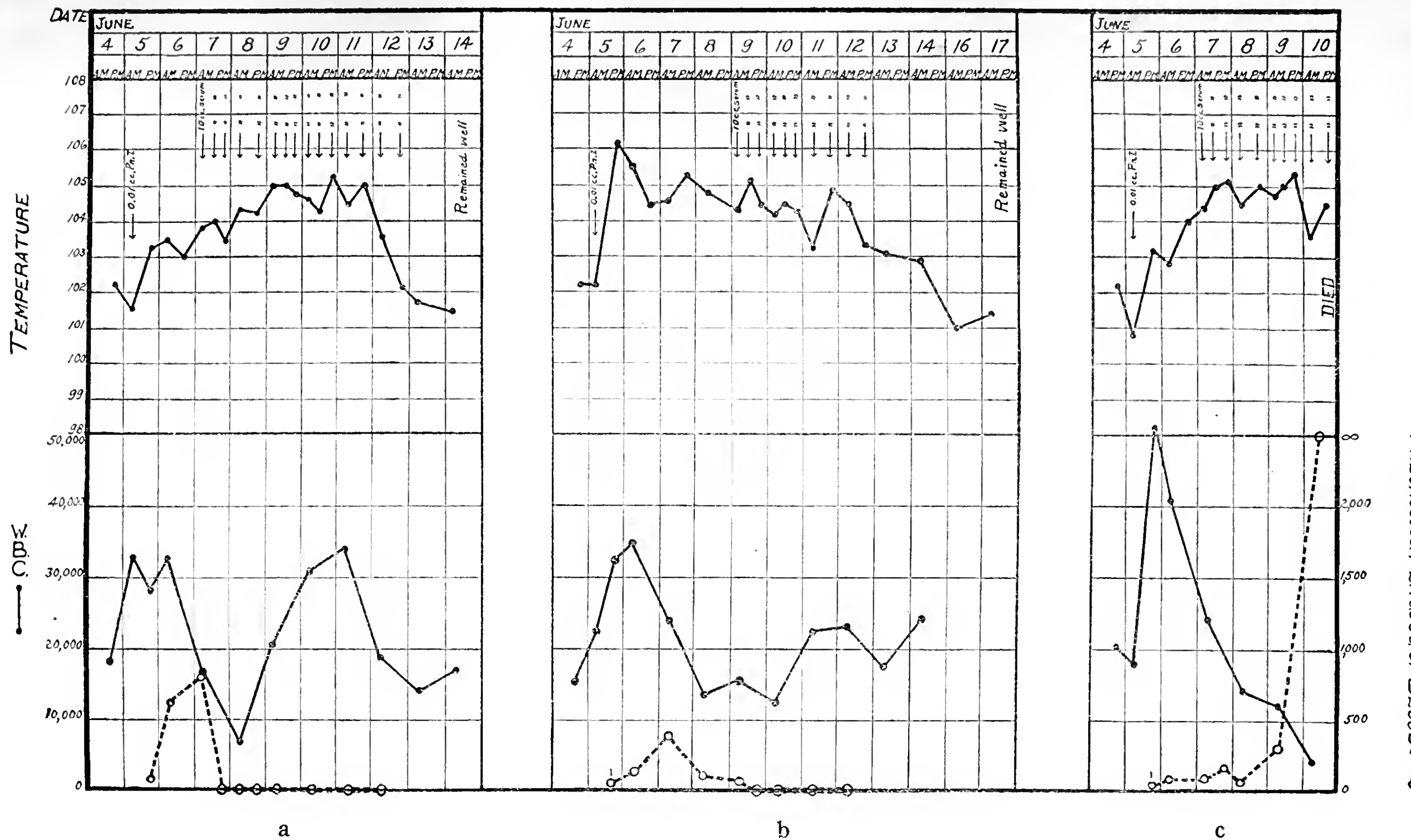
June 9. Sick; respirations rapid and labored. 10.25 a.m. Blood culture: 0.5 cc. in broth, Pneumococcus Type I; 0.5 cc. in agar plate, 17 colonies of Pneumococcus Type I. 10.30 a.m. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution. 4.15 p.m. Appears better. Blood culture: 0.5 cc. in broth, no growth; 0.5 cc. in agar plate, no growth. 4.20 p.m. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution. 9.30 p.m. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution.

June 10. Appears better and fairly active; temperature still elevated and respirations moderately rapid. 10.15 a.m. Blood culture: 0.5 cc. in broth, no growth; 0.5 cc. in agar plate, no growth. 10.20 a.m. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution. 4.20 p.m. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution. 9.30 p.m. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution.

June 11. Monkey appears much better; respirations still slightly accelerated. 10.15 a.m. Blood culture: 0.5 cc. in broth, no growth; 0.5 cc. in agar plate, no growth. 10.20 a.m. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution. 4.20 p.m. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution.

June 12. Appears in good condition. 10.15 a.m. Blood culture: 0.5 cc. in broth, no growth; 0.5 cc. in agar plate, no growth. 10.20 a.m. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution. 4.10 p.m. In excellent condition; temperature falling. Intravenous injection of 10 cc. of Antipneumococcus Serum Type I plus 10 cc. of normal saline solution.

June 13, 14, 17, 24. Remains well.



TEXT-FIG. 3, *a*, *b*, and *c*. Experimental Pneumococcus Type I pneumonia, showing the effect of Type I antipneumococcus serum when started late in the disease. (*a*) Monkey 99; treated with Type I antipneumococcus serum. (*b*) Monkey 113; treated with Type I antipneumococcus serum. (*c*) Monkey 112 (control); treated with normal horse serum.

These two protocols show that even when serum treatment is started late in the disease the life of the monkey may still be saved. The number of injections necessary, however, to achieve success was much larger than in the previous experiments in which treatment was started earlier. Monkey 99 received 0.01 cc. of broth culture of Pneumococcus Type I intratracheally and promptly developed pneumonia. 48 hours after the inoculation serum treatment was started, in injections of 10 cc. each, administered two or three times each day. This treatment was kept up for 6 days before the temperature finally became normal. It will be seen, however, that in this experiment as in the previous ones, the blood, which was heavily infected when treatment was started, became immediately sterile after the institution of serum treatment. In Monkey 113 the procedure was the same as with Monkey 99, except that serum treatment was delayed until 96 hours (4 days) after the intratracheal injection of pneumococci. Serum treatment was continued 4 days before the temperature returned to normal. As the infecting dose in both these monkeys was well above the minimum lethal dose, there is every reason to believe that both monkeys would have died from the infection if serum treatment had not been employed. This opinion is well supported by the death of the control (Monkey 112). These experiments show that with delay in the institution of serum treatment so prompt a cure cannot be expected as when treatment is started early in the disease, and they emphasize, therefore, very strongly the importance and value of early treatment.

Effect of Normal Horse Serum.—The control in this experiment (Monkey 112) was treated with normal horse serum in order to determine whether non-specific factors played any part in the favorable results obtained with antipneumococcus serum. The monkey received 0.01 cc. of broth culture of Pneumococcus Type I intratracheally, and 48 hours later serum treatment was started, the injections paralleling those of Monkey 99.

Monkey 112 (Text-fig. 3). *Macacus syrichtus*, male; weight 3,975 gm.

June 4, 1919. Well and active.

June 5. Well and active. 10.50 a.m. Intratracheal injection of 0.01 cc. of 18 hour broth culture of Pneumococcus Type I (in 1 cc. of broth). 4.50 p.m. Blood culture: 0.5 cc. in broth, Pneumococcus Type I; 0.5 cc. in agar plate, 1 colony of Pneumococcus I.

June 6, 10.45 a.m. Moderately sick; respirations accelerated. Blood culture: 0.5 cc. in broth, Pneumococcus I; 0.5 cc. in agar plate, 52 colonies of Pneumococcus I.

June 7, 10.20 a.m. Monkey sick; respirations rapid and labored. Blood culture: 0.5 cc. in broth, Pneumococcus I; 0.5 cc. in agar plate, 51 colonies of Pneumococcus I. 10.22 a.m. Intravenous injection of 10 cc. of normal horse serum plus 10 cc. of normal saline solution. No reaction. 4.10 p.m. Blood culture: 0.5 cc. in broth, Pneumococcus I; 0.5 cc. in agar plate, 90 colonies of Pneumococcus I. 4.15 p.m. Intravenous injection of 10 cc. of normal horse serum plus 10 cc. of normal saline solution. 9.30 p.m. Sick; respirations rapid and labored. Blood culture: 0.5 cc. in broth, Pneumococcus I; 0.5 cc. in agar plate, 21 colonies of Pneumococcus I. 9.35 p.m. Intravenous injection of 10 cc. of normal horse serum plus 10 cc. of normal saline solution.

June 8. Sick; respirations rapid and grunting. 10.40 a.m. Blood culture: 0.5 cc. in broth, Pneumococcus I; 0.5 cc. in agar plate, 17 colonies of Pneumococcus I. 10.45 a.m. Intravenous injection of 10 cc. of normal horse serum plus 10 cc. of normal saline solution. 5 p.m. Intravenous injection of 10 cc. of normal horse serum plus 10 cc. of normal saline solution.

June 9. Very sick; respirations rapid and labored; dullness, bronchial breathing, and râles throughout right axilla. 10.45 a.m. Blood culture: 0.5 cc. in broth, Pneumococcus I; 0.5 cc. in agar plate, 305 colonies of Pneumococcus I. 10.50 a.m. Intravenous injection of 10 cc. of normal horse serum plus 10 cc. of normal saline solution. 4.30 p.m. Very sick. Intravenous injection of 10 cc. of normal horse serum plus 10 cc. of normal saline solution. 10 p.m. Very sick. Intravenous injection of 10 cc. of normal horse serum plus 10 cc. of normal saline solution.

June 10. Very sick; respirations rapid and labored. 10.30 a.m. Blood culture: 0.5 cc. in broth, Pneumococcus I; 0.5 cc. in agar plate, innumerable colonies of Pneumococcus I. 10.35 a.m. Intravenous injection of 10 cc. of normal horse serum plus 10 cc. of normal saline solution. 4.30 p.m. Intravenous injection of 10 cc. of normal horse serum plus 10 cc. of normal saline solution. 8.30 p.m. Died.

Autopsy.—Performed June 11, 9.30 a.m.

Anatomical Diagnosis.—Experimental lobar pneumonia (Pneumococcus Type I); red and gray hepatization of right middle and lower lobes; engorgement of left lower lobe; acute fibrinous pleuritis, right side; hypertrophy of the heart; cloudy swelling of viscera; Pneumococcus Type I septicemia.

Subject.—Large male *Macacus.* Postmortem rigidity is present. Abdominal organs show cloudy swelling but otherwise are normal. Pericardial and left pleural cavities are normal. There are a few fine fresh adhesions over the right lower lobe.

Heart.—Definitely enlarged and left ventricular wall appears thicker than normal. Myocardium and valves show no changes. *Right lung.*—Considerably

larger than the left, due to massive consolidation of the middle and lower lobes, and emphysema of upper lobe. The latter is pale and air-containing throughout. The middle and lower lobes are completely consolidated and are plastered together by fibrin. There is also a fibrinous exudate on the surface. The cut surface of the consolidated lobes is pinkish gray, mottled with patches of reddish hue, indicative of the transition from red to gray hepatization. *Left lung.*—Air-containing throughout, but the posterior portion shows marked hypostatic congestion. This is most marked in the lower lobe, the greater part of which presents a dark red color. A part of this congestion may be an expression of early infection; that is, of engorgement. *Trachea and bronchi.*—Contain blood-tinged mucus.

Microscopic Examination.—Right lower lobe.—Section shows an exudate of polymorphonuclear leucocytes which almost completely fills the alveoli. In some places the exudate also contains many red blood cells. In a few places resolution is beginning, as is indicated by the desquamation of epithelium and the disintegration of the leucocytes. Sometimes the dissolution of the cells is so advanced that practically nothing remains but an amorphous residue. There is little or no perivascular infiltration of leucocytes, but there is a very noticeable extravasation of red blood cells in the perivascular tissue. The bronchi contain pus; the bronchial epithelium has been destroyed in some places. Section stained for bacteria shows a moderate number of pneumococci in the alveoli. Many of these, however, stain poorly. *Left lower lobe.*—Section shows engorgement of all the blood vessels, including the capillaries. There is a moderate amount of interstitial infiltration of leucocytes in some places. A few pneumococci are seen here and there in the alveolar walls.

Autopsy Cultures.—Heart's blood, Pneumococcus I; right lower lobe, Pneumococcus I.

It is obvious from a study of this protocol that normal horse serum had no beneficial effect whatever on the pneumococcus septicemia, which gradually increased in severity; nor did it have any apparent effect on the course of the pneumonia. The monkey died on the 6th day of the disease, in spite of ten injections of horse serum.

DISCUSSION.

Little discussion is needed in connection with the experiments which have just been described. They prove without any reasonable doubt that Type I antipneumococcus serum has a highly specific therapeutic action on Pneumococcus Type I pneumonia. This action is most rapidly effective when the serum is administered early in the disease. At this stage intravenous injection of Type I antipneumo-

coccus serum at frequent intervals will completely abort what would otherwise be a serious and often fatal disease. These experiments also show that even when started late in very severe cases serum treatment may cure the disease, though without appreciably shortening its course.

Failure to obtain favorable results with antipneumococcus serum can usually be charged to one or more of several causes: (1) Incorrect determination of the type of pneumococcus. Pneumococcus Type I serum is highly specific for Pneumococcus Type I pneumonia, and probably has no beneficial effect whatever when used in other types of pneumonia. (2) The serum treatment is started too late in the disease. This is one of the most frequent mistakes made in connection with serum treatment of pneumonia. When this delay is unavoidable, however, the present studies have shown that it is always worth while to use the serum late in the disease, though in such cases a larger number of injections may be necessary. (3) The serum is administered in too small doses. This mistake was often made in the Army camp hospitals where only 40 or 50 cc. of serum were injected at one time. (4) The serum is not given at sufficiently frequent intervals. Cole recommends that serum be administered every 8 hours. As a matter of practice, however, in many hospitals, serum is given only once or twice in 24 hours and sometimes not that often.

In the summer of 1918 there was a small epidemic of Pneumococcus Type I pneumonia among the negro troops at Camp Wheeler in which the mortality rate was surprisingly high. In a series of twenty-nine cases of Pneumococcus Type I pneumonia that received Type I antipneumococcus serum, there were ten deaths, or a mortality rate of 34.5 per cent. As the mortality rate of Type I pneumonia in cases treated with serum was only 7 per cent in the Rockefeller Institute series, it was evident that there was some defect in the method of administering the serum at Camp Wheeler. An investigation made by one of us showed that two-thirds of these cases did not receive serum until 48 hours or more after admission to the hospital. One-half of the cases did not receive the first injection of serum until at least 4 days after admission. The average number of serum injections was three, and the total amount of serum given

each patient averaged about 175 cc. From these figures it would appear that at Camp Wheeler antipneumococcus serum was not administered early enough, frequently enough, or in sufficiently large doses, to be of therapeutic value.

Finally, it should be pointed out that two of the monkeys in the experiments (Nos. 45 and 79) received doses of serum about three times as large proportionately as human patients receive in our hospitals, and suffered no apparent ill effects therefrom. This brings up the question whether even more brilliant results could not be obtained with serum treatment in man, if the routine dose of antipneumococcus serum were larger. It would have to be determined, of course, whether this modification could be introduced without discomfort or danger to the patient.

CONCLUSIONS.

1. In experimental Pneumococcus Type I pneumonia in monkeys the intravenous injection of Type I antipneumococcus serum exercises a specific therapeutic effect, frees the blood promptly and permanently from pneumococci, shortens the course of the disease, and greatly moderates its severity. Of five monkeys inoculated intratracheally with lethal doses of Pneumococcus Type I, all developed pneumonia, and all recovered following the administration of Type I antipneumococcus serum, while the controls died.

2. The earlier the serum is administered the shorter and less severe the pneumonia. Frequent injections are also an important factor in obtaining favorable results. When serum treatment is instituted late in the disease, the injections must usually be continued over a longer period of time in order to achieve success.

3. Normal horse serum exerts no beneficial effect whatever in experimental Pneumococcus Type I pneumonia.

A GROUP OF PARATYPHOID BACILLI FROM ANIMALS CLOSELY RESEMBLING THOSE FOUND IN MAN.

By CARL TENBROECK, M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Received for publication, March 5, 1920.)

In a previous paper (1) the writer described a group of paratyphoid bacilli, isolated from animals, that with the usual cultural methods seemed to be the same as paratyphoid B. or *Bacillus schottmülleri*, as Winslow, Kligler, and Rothberg (2) suggest we call the latter organism. Serologically they could be separated from one another by agglutination absorption tests and to a certain extent by the type of clumps formed in immune sera. Five new cultures belonging to this group have recently been studied and a further effort has been made to differentiate them from the human paratyphoids by a method less complicated than agglutination absorption.

Five of the cultures studied came from swine infected with hog-cholera virus, two came from guinea pigs, and one each from a child, a cow, a pigeon, and a mouse. Four cultures of *Bacillus schottmülleri* obtained from Dr. C. Krumwiede were used for comparison. The date of isolation and other facts regarding the above cultures are given in the paper already referred to. In addition, cultures isolated by Dr. Smith in 1918 from five calves have been studied and found to belong to the same group, and still more recently additional cultures of this group have been isolated from swine infected with hog-cholera virus. It is evident that these organisms are quite widespread among our domestic animals but just what relation they have to animal diseases will require much further study. In hog-cholera they may tend to emphasize the intestinal lesions, but cases of this disease occur with extensive intestinal changes from which organisms of this group cannot be isolated.

Cultural Studies.

Sixteen cultures of the animal paratyphoids have been studied culturally and they have all corresponded to one another and to *Bacillus schottmülleri.* The results of the study are given in Table I, which includes the characters of *Bacillus enteritidis* and of the hog-cholera bacillus.

Some of the strains were tested with inosite with unsatisfactory results as the amount of acid produced by the paratyphoids was so small that it seemed possible that it was due to impurities. Other carbohydrates might have been included in the study but they are so

TABLE I.

Cultural Characters of Paratyphoids Studied.

Organisms.	Motility.	Gram stain.	Indole.	Hydrogen sulfide.	Gelatin liquefied.	Dextrose.	Maltose.	Mannitol.	Xylose.	Dulcite.	Arabinose.	Lactose.	Saccharose.	Salicin.	Glycerol.	Raffinose.	Dextrin.
B. schottmülleri (paratyphoid B).	+	–	0	+	0	AG*	AG	AG	AG	AG	AG	0	0	0	0	0	0
B. enteritidis.	+	–	0	+	0	"	"	"	"	"	"	0	0	0	0	0	0
Animal paratyphoids.	+	–	0	+	0	"	"	"	"	"	"	0	0	0	0	0	0
Hog-cholera bacilli.	+	–	0	0	0	"	"	"	"	0	0	0	0	0	Slightly acid.	0	0

* AG indicates acid and gas formation.

expensive that they would be of no practical value in differentiating the two groups. As shown in the table, no cultural differences have been found between *Bacillus enteritidis, Bacillus schottmülleri,* and the animal paratyphoids.

In the hope that a difference might be detected between animal and human paratyphoids, by a study of the hydrogen ion concentration, cultures were made in dextrose and xylose broth. Both groups gave a hydrogen ion concentration of pH 5.0 in dextrose broth after 4 days incubation, while in xylose broth the pH varied from 5.6 to 5.2. Some of the animal strains acted more slowly and others apparently utilized more of the carbohydrate than did the human strains. The difference is so slight, however, that it is of no value in classification.

Serological Studies.

When living cultures are used for immunization of rabbits the sera produced will usually agglutinate both the human and animal paratyphoids to the same titer limit. At times, however, one gets a serum that will agglutinate bacilli of the group used in the immunization in higher dilutions than it does those of the other group. This is apparently due to the individual rabbit rather than the bacteria, for the same culture will act differently in different rabbits.

Cultures from both human and animal sources are agglutinated in only the lower dilutions of sera from animals immune to *Bacillus enteritidis* and as the results obtained in the present study do not differ from those previously given they need not be repeated here.

When 24 hour bouillon cultures are used as antigens the clumps formed by bacilli of the same group as the immunizing strain are flocculent and after 2 hours incubation form a mass occupying from one-quarter to one-half the column of liquid. Bacilli of the other group as a rule give very compact clumps which after standing in the refrigerator over night form a thin film on the bottom of the test-tube. This difference in the clumping is striking but unfortunately it does not always hold true. If growth is more abundant than the average, flocculent clumping may occur where a compact type is expected. The amount of dextrose in the bouillon influences the type of clumps formed. Apparently the amount of growth rather than the change in reaction is responsible for the change. As a rule, however, one can differentiate fairly well between the two groups by the type of clumps, but it can only be regarded as a tentative means of differentiation.

When heated or formalinized bouillon cultures or suspensions in salt solution of the growth from agar slants are used as antigens, agglutination will occur in the same dilutions of serum and the clumps are so nearly alike in character that the two groups cannot be differentiated.

When formalinized bouillon cultures are standardized and agglutinated according to Dreyer's method (3) with the readings made after 2 hours incubation at 50°C. in the water bath and 15 minutes at room temperature, the two groups cannot be differentiated by the degree of agglutination or the type of clumps.

Numerous agglutination absorption tests have been made and the results have confirmed those previously reported. The animal strains will absorb from *Bacillus schottmülleri* sera the agglutinin for all the

TABLE II.

Summary of Absorption Experiments with Serum of Rabbit 9, Immunized to Calf-Typhus V by Injection of Living Cultures.

Culture tested.	Titer limit of serum saturated with.					
	Nothing; *i.e.*, control.	*B. schottmülleri* 232.	*B. schottmülleri* 242.	Calf-typhus V.	Swine-typhus I.	Swine-typhus V.
Calf-typhus I	51,200	25,600	25,600	—	—	—
" III	51,200	—	—	—	400	400
" IV	51,200	25,600	25,600	800	—	—
" V	51,200	25,600	25,600	200	400	400
Swine-typhus I	51,200	12,800	12,800	—	200	—
" V	51,200	25,600	25,600	—	—	200
Pigeon-typhus	51,200	25,600	25,600	—	—	—
Mouse-typhus I	51,200	25,600	25,600	—	—	—
Hog-cholera XII	51,200	400	200	—	—	—
" XIII	51,200	400	200	—	—	—
B. schottmülleri 232	51,200	400	200	100	—	—
" " 242	51,200	400	400	—	—	—
" " 225	51,200	200	800	100	—	—

TABLE III.

Absorption of Agglutinins from B. schottmülleri Serum by Calf-Typhus Cultures.

Culture tested.	Titer limit of serum of Rabbit 10, immune to *B. schottmülleri* 232, absorbed with.		
	Nothing; *i.e.*, control.	Calf-typhus V.	Calf-typhus I.
Calf-typhus I	12,800	200	400
" IV	12,800	100	100
" V	12,800	200	400
Swine-typhus I	12,800	200	400
B. schottmülleri 225	25,600	25,600	25,600
" " 232	25,600	25,600	25,600

animal cultures without removing those for the human cultures, and, *vice versa*, *Bacillus schottmülleri* will remove from the sera of animals immune to the animal cultures the agglutinin for the human cultures

and will not remove those for the animal strains. The calf and swine cultures are identical in this respect as is shown in Tables II and III.

While the great majority of the agglutination tests was made with sera of animals immunized by the injection of living bouillon cultures, the possibility that sera produced by the injection of heated cultures might differentiate the two groups has also been considered. The serum of an animal immunized by three injections of a suspension of agar slant growth, killed by heating to 60°C. for 1 hour, agglutinated both groups of bacilli to the same degree. When sera of animals immunized by bacilli heated to 70° for 1 hour were tested, it was found that the bacilli of the immunizing group were agglutinated in a somewhat higher dilution than were those of the other group. The difference was not great enough to make it a valuable means of differentiation. Better results might be obtained by using heated and washed bacilli, but this has not been tried.

Since the type of clumping indicates that the difference in these two groups lies in the flagella, antigens were prepared by shaking suspensions of the bacilli for a short time, centrifugalizing, and using the supernatant fluid for precipitation and complement fixation tests. When the antigen belonged to the same group as the immune serum, the precipitate was more flocculent than when it belonged to the other group, but the amount of precipitation was about the same. When such antigens were used for complement fixation, the inhibition of hemolysis was the same with sera of rabbits immune to either group. Formalinized bouillon cultures used as antigens in complement fixation tests were likewise of no value in differentiating the two groups. These results agree with those of the previous paper where extracts of the bacilli were used as antigens.

The results of the serological tests show that the animal paratyphoids which are usually agglutinated to the titer limit in sera of animals immune to *Bacillus schottmülleri* can best be separated from the latter by agglutination absorption tests. With the methods commonly used in agglutination tests they would be classed as *Bacillus schottmülleri*.

Cross-Immunization Tests.

In the previous paper it was noted that the injection of living cultures of the swine paratyphoids immunized rabbits to a virulent hog-cholera bacillus, whereas rabbits treated in the same way with living cultures of human paratyphoid were not immune. This seemed at that time to indicate that the swine cultures were more closely related to, or possibly were a variety of, the hog-cholera bacillus. These tests have been repeated and the results are given in Table IV.

It will be seen that the calf cultures also immunize rabbits to the hog-cholera bacillus, whereas the human cultures do not. Examination of the records shows that the injection of the animal cultures produces a more severe type of reaction than does the injection of the human cultures. The local lesion is larger and the rise in temperature following a subcutaneous reaction is higher.

Two rabbits were each given a subcutaneous injection of 0.1 cc. of a 24 hour bouillon culture of two strains of human paratyphoid and were chloroformed 1 week later. There was a slight local lesion from which the organisms injected were cultured. The spleen and other organs were normal and cultures from as much as 0.5 cc. of blood, a piece of liver as large as a pea, and stab cultures from the spleen were sterile. Pea-sized bits of spleen from one rabbit showed no organisms, while from the other there was a growth which proved to be due to the organism injected. 1 week after an intravenous injection of 0.1 cc. of 24 hour bouillon cultures, two other rabbits were chloroformed and their spleens were found to be slightly enlarged. Cultures from the spleen of one of these rabbits showed a growth due to the organism injected, whereas the spleen of the other animal failed to show such organism. The blood, liver, and bile of both rabbits were sterile.

These results show that there is very little growth of the human paratyphoid bacilli in rabbits when they are used in the same amounts that will produce a general invasion by the animal cultures. It therefore seems probable that the immunity to the hog-cholera bacillus produced by the animal paratyphoids is due to the fact that they multiply in the body and increase the resistance enough so that the animals are able to withstand the hog-cholera bacilli injected, when the amount of the latter used is about ten times the minimal lethal dose.

TABLE IV.

Test of Power of Various Paratyphoids to Immunize Rabbits to a Virulent Hog-Cholera Bacillus Culture.

Rabbit No.	Inoculated with.	1st injection.	2nd injection.	3rd injection.	Weight Nov. 18.	Result of subcutaneous injection of 0.000001 cc. of 24 hr. bouillon culture of Hog-cholera XII. Rabbit series, Nov. 18 1919.
		1919	*1919*	*1919*	*gm.*	
1	Swine-typhus II, bouillon culture.	Oct. 4. 0.1 cc. subcutaneously.	Oct. 25. 0.01 cc. intravenously.	—	2,523	Lived.
2	Swine-typhus IV, bouillon culture.	Oct. 4. 0.1 cc. subcutaneously.	Oct. 25. 0.01 cc. intravenously.	—	2,700	"
3	Calf-typhus I, bouillon culture.	Oct. 4. 0.1 cc. subcutaneously.	Oct. 25. 0.01 cc. intravenously.	—	2,164	"
4	Calf-typhus III, bouillon culture.	Oct. 4. 0.1 cc. subcutaneously.	Oct. 25. 0.01 cc. intravenously.	—	2,684	"
5	*B. schottmülleri* 232, bouillon culture.	Oct. 4. 0.1 cc. subcutaneously.	Oct. 25. 0.01 cc. intravenously.	—	2,509	Death in 7 days.
6	*B. schottmülleri*, 242, bouillon culture.	Oct. 4. 0.1 cc. subcutaneously.	Oct. 25. 0.01 cc. intravenously.	—	2,561	" " 10 "
7 Control.	Calf-typhus III, suspension of agar growth, heated 1 hr. at 70°.	Oct. 17. 0.5 cc. subcutaneously.	Oct. 24. 1 cc. subcutaneously.	Nov. 1. 2 cc. subcutaneously.	2,520	" " 9 "
8 Control.	Swine-typhus II, suspension of agar growth, heated 1 hr. at 70°.	Oct. 17. 0.5 cc. subcutaneously.	Oct. 24. 1 cc. subcutaneously.	Nov. 1. 2 cc. subcutaneously.	2,752	" " 6 "
9 Control.	No previous treatment.	—	—	—	2,453	" " 8 "

The intimate relation of these organisms is shown by cross-immunization tests made with mice. These animals were immunized at the same time by subcutaneous injections of 0.0001 cc. followed by intraperitoneal injections of 0.005 cc. of 24 hour bouillon cultures. After they had recovered from the latter injection their immunity towards other cultures was tested by intraperitoneal injections of 0.005 cc. of 24 hour bouillon cultures. The results of the test are given in Table V and show that the animal and human cultures immunize against one another and that the calf culture immunized against the swine and *vice versa*.

TABLE V.

*Test of Cross-Immunity of Mice to Paratyphoids of Other Animals.**

Mice immune to.	Result of intraperitoneal injection of 0.005 cc. of 24 hr. bouillon culture.					
	Calf-typhus V.		*B. schottmülleri* 232.		Swine-typhus V.	
	Lived.	Died.	Lived.	Died.	Lived.	Died.
Calf-typhus V	4	0	4	0	4	0
B. schottmülleri 232	3	1	4	0	4	0
Swine-typhus V	3	0	3	0	3	0
Controls. No previous treatment	0	4	1	3	0	4

* See text for methods of immunization.

DISCUSSION.

The question arises whether we should place these animal paratyphoids, that culturally are the same as *Bacillus schottmülleri*, in a separate group because they fail to absorb the agglutinins from the serum of animals immune to the latter. This failure to absorb agglutinins seems to be a very fundamental difference and these organisms should be regarded as a distinct variety of paratyphoid. No differences either cultural or serological have been detected between the strains derived from swine, calves, and the few strains from the other species that have been studied.

A common name is desirable for this group of organisms and if it is found, upon further study, that these organisms are the same as the

Aertrycke bacillus, isolated by de Nobele (4) from an outbreak of food poisoning, the name of *Bacillus aertryckei* would be appropriate. It is possible that there is one host that harbors these organisms and from it the other animals become infected. If this should prove to be the case, a name indicating this host would be the logical one. On the other hand, the naming of the animal from which a particular culture is isolated is desirable and for the present I propose that they be called typhus with a prefix denoting the animal from which they were isolated. The name typhus has only its long use to commend it, as the organisms are not like *Bacillus typhosus*, nor do they produce a disease that very closely resembles typhoid fever. Another objection is that in the cultures from the smaller animals the word typhus has been used in connection with the paratyphoid disease that is so common. In my experience most of these cultures from mice and guinea pigs belong to the *enteritidis* group and can be separated by their specific agglutination characters, though organisms of the group under consideration also occur. *Bacillus enteritidis* also occurs in the larger animals. I have found it in one pig, Jensen (5), Meyer, Traum, and Roadhouse (6), and others have found it in calves with diarrhea, and Graham, Reynolds, and Hill (7) have found it in an acute disease of horses. In the future it would be well to call these *Bacillus enteritidis* and reserve the use of mouse-typhus, guinea pig-typhus, etc., for the organisms that we have considered in this paper.

It is evident that the group that has been considered in this paper has been encountered before. I have already (1) pointed out that many of the strains of so called *Bacillus suipestifer* probably belong to this group. Bock (8) noted that when he saturated the sera of animals immune to mouse-typhus, *Bacillus suipestifer*, or bacilli obtained from outbreaks of food poisoning with *Bacillus schottmülleri* the agglutinins for the last bacillus were removed while those for the first three organisms were not affected. Sobernheim and Seligmann (9) in studying paratyphoid bacilli noted that three cultures classed as *Bacillus schottmülleri* formed fine clumps in the serum of an animal immune to *Bacillus schottmülleri*. They immunized a rabbit to one of these strains heated to 70°C. and found that the serum agglutinated the three cultures to the same degree while the other *Bacillus schottmülleri* cultures were agglutinated in only the lower dilutions of serum. Especially significant

are the observations of Bainbridge and O'Brien (10). They compared cultures from cases of food poisoning with those from undoubted cases of paratyphoid fever. In *Bacillus schottmülleri* sera the former produced fine clumps and did not absorb the agglutinins for the immunizing strains, whereas the latter formed flocculent clumps and absorbed all the agglutinins from the serum. Their control cultures of *Bacillus suipestifer* acted the same as those from food poisonings, but as these controls were obtained from German laboratories it is probable that they were the same as those I have called swine-typhus. Krumwiede, Valentine, and Kohn (11) separated from *Bacillus schottmülleri* by absorption tests a number of organisms obtained from rodents which probably belong to this group.

As noted above, when suspensions of agar slant growth or killed cultures are used as antigens in agglutination tests the difference between these animal cultures and *Bacillus schottmülleri* could not be detected and it seems possible that the so called paratyphoid B bacilli that Jensen (5), Christiansen (12), and others have associated with diarrhea in calves are the same as the organisms I have called calf-typhus. Many of the paratyphoid B bacilli isolated from food poisonings quite possibly belong to the same group but from the literature one cannot draw any conclusions as the diagnosis has usually been made on the agglutination test without supplementary absorption tests. This subject is important because we want to know what type of infections man gets from the lower animals.

There are several well recognized groups of pathogenic animal paratyphoids besides *enteritidis* and the group considered here. The hog-cholera bacillus, or better, *Bacillus choleræ suis*, described by Smith (13), differs from the others by being highly pathogenic for rabbits. More recently (1) it has been shown to have distinct cultural differences from *Bacillus schottmülleri*. The Voldagsen bacillus described by Dammann and Stedefeder (14) and the "Ferkel typhus" bacillus described by Glässer (15) seem to be identical. They are differentiated from the other paratyphoids by their failure to act on mannitol and the fact that they produce little or no gas. *Bacillus abortus equi*, first described by Smith and Kilborne (16) and later studied by Meyer and Boerner (17) and others, resembles *Bacillus schottmülleri* culturally, except that it fails to produce hydrogen sulfide, and on agar forms a

dry brittle growth. Serologically Meyer and Boerner, and Murray (18) place it in a group by itself. In its virulence for rabbits Smith and Kilborne pointed out that it resembled a mildly virulent hog-cholera bacillus.

Jordan (19) and Reerstorp (20) have found a variety of so called intermediate paratyphoids in the intestinal tract of normal swine, and from children Lewis (21) and others have obtained paratyphoids which have been classified culturally by Graham-Smith (22). It is difficult to determine just what relation these paratyphoids of the normal digestive tract bear to the established groups. They differ serologically and culturally from the members of these several groups but it is conceivable that under certain conditions they might invade the body and change their characters.

Smith and Reagh (23) discussed the possibility of the host changing the agglutinative characters of organisms and such a possibility should be considered here. After passing from animal to man and becoming adapted to the latter, it is quite possible that organisms might change both their cultural and agglutinative characters. Careful study of food poisoning outbreaks due to eating meat containing these animal paratyphoids might throw some light on this subject. If such a change does occur it would result in much confusion. I have from time to time modified slightly the cultural characters of some of these paratyphoids by passage through animals, but on the whole the cultural and especially the agglutination characters are remarkably constant.

CONCLUSIONS.

1. In addition to the paratyphoid bacilli already named there exists a group which occurs in a variety of animals and which culturally is the same as *Bacillus schottmülleri*. As a rule this group can be separated from the latter by the type of clumps formed when bouillon cultures are used as antigens, while other antigens and complement fixation tests have failed to differentiate it. Agglutination absorption tests sharply separate the animal from the human paratyphoids.

2. No differences have been detected between organisms of this group derived from a number of animals and a common name for them is desirable, but for the present it seems better to call them calf-,

swine-, mouse-, etc., typhus, according to the animal from which they were isolated.

3. Evidence exists in the literature that these organisms have been associated with food infections in man, particularly with what have been called paratyphoid B infections, but this function, as well as the part they play in animal diseases, is a subject for further study.

4. Well defined groups of paratyphoid such as *Bacillus choleræ suis*, the Voldagsen bacillus, *Bacillus abortus equi*, and *Bacillus enteritidis* are found in animals in addition to the organisms considered in this paper, and every attempt should be made to range newly isolated organisms in one or the other of these well recognized groups.

5. One of the objects in continuing this work was to find a method of differentiating these animal from the human paratyphoids less complicated than agglutination absorption. This object was not realized; the two groups are very similar and agglutination absorption seems to be the only means of classifying them.

The writer is grateful to Mr. Henry Hagens of this Laboratory for valuable technical assistance.

BIBLIOGRAPHY.

1. TenBroeck, C., *J. Exp. Med.*, 1918, xxviii, 759.
2. Winslow, C.-E. A., Kligler, I. J., and Rothberg, W., *J. Bacteriol.*, 1919, iv, 429.
3. Dreyer, G., quoted by Gardner, A. D., *J. Hyg.*, 1918, xvii, 471.
4. de Nobele, J., *Ann. Soc. méd. Gand*, 1898, lxxvii, 281; 1901, lxxx, 74.
5. Jensen, C. O., in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1913, vi, 121.
6. Meyer, K. F., Traum, J., and Roadhouse, C. L., *J. Am. Vet. Med. Assn.*, 1916, xlix, 17.
7. Graham, R., Reynolds, F. H. K., and Hill, J. F., *J. Am. Vet. Med. Assn.*, 1920, lvi, 378, 489, 586.
8. Bock, F., *Arb. k. Gsndhtsamte*, 1906, xxiv, 237.
9. Sobernheim, G., and Seligmann, E., *Z. Immunitätsforsch., Orig.*, 1910, vi, 401.
10. Bainbridge, F. A., and O'Brien, R. A., *J. Hyg.*, 1911, xi, 68.
11. Krumwiede, C., Jr., Valentine, E., and Kohn, L. A., *J. Med. Research*, 1918–19, xxxix, 449.
12. Christiansen, M., *Centr. Bakteriol., 1te Abt., Orig.*, 1917, lxxix, 196.
13. Smith, T., Hog cholera: its history, nature, and treatment, *U. S. Dept. Agric., Bureau Animal Industry*, 1889, 69.

14. Dammann, K., and Stedefeder, K., *Arch. wissensch. u. prakt. Tierheilk.*, 1910, xxxvi, 432.
15. Glässer, K., *Deutsch. tierärztl. Woch.*, 1908, No. 40, 569.
16. Smith, T., and Kilborne, F. L., *U. S. Dept. Agric., Bureau Animal Industry, Bull. 3*, 1893, 49, 53.
17. Meyer, K. F., and Boerner, F., *J. Med. Research*, 1913, xxix, 325.
18. Murray, C., *J. Infect. Dis.*, 1919, xxv, 341.
19. Jordan, E. O., *J. Infect. Dis.*, 1918, xxii, 252.
20. Reerstorp, A. R., *Den k. Vet. Landbohφiskoles Aarsskrift*, 1919, 113.
21. Lewis, C. J., *Gt. Britain Local Gov. Bd., Suppl. Rep. Med. Off.*, 1910–11, xl, 314.
22. Graham-Smith, G. S., *Gt. Britain Local Gov. Bd., Suppl. Rep. Med. Off.*, 1911–12, xli, 304.
23. Smith, T., and Reagh, A. L., *J. Med. Research*, 1903, ix, 270.

BACILLI OF THE HOG-CHOLERA GROUP (BACILLUS CHOLERÆ SUIS) IN MAN.

By CARL TENBROECK, M.D.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

(Received for publication, March 5, 1920.)

Hirschfeld (1) has described an epidemic of clinical paratyphoid fever which occurred in Serbia or Greece from which he obtained organisms culturally paratyphoid B or *Bacillus schottmülleri*, but which were not agglutinated by the sera of animals immune to the latter bacillus. These organisms were isolated eighteen times, twice being obtained after death. Sera obtained from patients in this epidemic agglutinated the organisms isolated in some cases in dilutions as high as 1:800. MacAdam (2) obtained similar inagglutinable paratyphoid bacilli in Mesopotamia from the blood stream of patients who clinically showed respiratory rather than enteric symptoms. Mackie and Bowen (3) have described cultures of the same group, as shown by Schütze's (4) work. The latter compared twelve cultures obtained from febrile cases occurring in the Balkan region, the organism isolated by the above writers being included. All the cultures were agglutinated by and absorbed the agglutinin from the serum of an animal immune to one of Hirschfeld's strains. Hirschfeld called his organisms paratyphoid C and Schütze called them Hirschfeld's bacillus.

It seemed possible that by comparing these organisms with the paratyphoids found in animals they might be classed in one of the known groups. Upon request Dr. Hirschfeld kindly sent me two cultures of these organisms and some immune serum. The cultures were labeled Para C and it may be that they were duplicates but I have called them Paratyphoid C I and II and have used both of them in the tests made. No differences have been detected between the two strains.

Agglutination tests were made with the serum received and the results given in Table I were obtained.

The serum agglutinates two strains of the hog-cholera bacillus in as high dilution as it does the organism isolated by Hirschfeld, while *Bacillus schottmülleri*, *Bacillus enteritidis*, and swine-typhus bacilli are agglutinated in only the lowest dilutions.

Anti-hog-cholera bacillus serum was absorbed with the cultures received and the results of agglutination tests with the absorbed as well as the unabsorbed serum are given in Table II.

The paratyphoid bacilli under consideration are agglutinated to the titer limit by anti-hog-cholera bacillus serum and absorb from this serum not only the agglutinins for themselves but also those for the hog-cholera bacilli.

TABLE I.

Agglutination with Hirschfeld Paratyphoid C Serum.

Culture tested.	Limit of agglutination.
B. schottmülleri 232	100
" " 242	100
Swine *enteritidis* I	50
B. enteritidis (Mt. Sinai)	50
Swine-typhus I	100
" V	100
Hog-cholera XII	12,800
" XIV	12,800
Paratyphoid C I	12,800
" " II	12,800

TABLE II.

Tests with Anti-Hog-Cholera Bacillus Serum.

Culture tested.	Limit of agglutination using serum.		
	Unabsorbed.	Absorbed with Paratyphoid C I.	Absorbed with Paratyphoid C II.
Paratyphoid C I	12,800	200−	200−
" " II	12,800	200−	200−
Hog-cholera bacillus XII	12,800	200	200
" " XVI	12,800	200	400

Rabbits were immunized, one to each of the two strains, by the injection of unheated bouillon cultures and their sera gave the results in Table III.

Four different hog-cholera bacillus cultures were agglutinated to the titer limit. Three of the hog-cholera bacillus cultures were used for absorption tests, one for one serum and two for the other, and they

took out the agglutinins for the Paratyphoid C bacilli, as well as for the other hog-cholera bacilli.

Serologically these organisms are typical hog-cholera bacilli but culturally they differ in that they form acid and gas in dulcite and arabinose and form hydrogen sulfide, whereas the hog-cholera bacillus, as has been pointed out by Jordan (5) and Krumwiede, Kohn, and Valentine (6), do not act on these carbohydrates or form hydrogen sulfide. Culturally, then, they are the same as *Bacillus schottmülleri.*

Two rabbits given subcutaneous injections of 0.1 cc. of 24 hour bouillon cultures of these organisms showed a slight rise in temperature

TABLE III.

Saturation of Paratyphoid C Sera with Hog-Cholera Bacilli.

Culture tested.	Limit of agglutination with serum of Rabbit A, immune to Paratyphoid C I.		Limit of agglutination with serum of Rabbit B, immune to Paratyphoid C II.		
	Unabsorbed.	Absorbed with Hog-cholera bacillus XVI.	Unabsorbed.	Absorbed with Hog-cholera bacillus X.	Absorbed with Hog-cholera bacillus XII.
Hog-cholera X, Pig 30	12,800	200	12,800	400	200−
" XI	12,800	200−	12,800	400	200−
" XII	12,800	200−	12,800	800	800
" XVI	12,800	200−	12,800	400	400
Paratyphoid C I	12,800	200−	12,800	400	800
" " II	12,800	200−	12,800	400	800

and local lesions. Neither was very sick and recovery was prompt. A typical hog-cholera bacillus should kill rabbits in from 6 to 10 days, so that these cultures resemble the animal typhus group rather than the hog-cholera bacilli in their virulence for rabbits.

20 days after the subcutaneous injection these rabbits were given an intravenous injection of 0.01 cc. of 24 hour bouillon cultures of the same organisms. This produced no effect and 25 days later they were given a subcutaneous injection of 0.00001 cc. of a 24 hour bouillon culture of a virulent hog-cholera bacillus. This organism in a dilution 100 times the one used here will kill normal rabbits in from 6 to 10 days, but in these injected rabbits it produced no rise in temperature, loss in weight, or local lesion. The rabbits were

killed 6 weeks after the final injection and aside from a slight enlargement of the spleen appeared normal. Cultures from the spleen showed hog-cholera bacilli from one rabbit but not from the other.

The interpretation of this experiment is not clear. As has been pointed out in the preceding paper (7), various animal typhus cultures will immunize rabbits to this virulent hog-cholera bacillus, but no tests have been made with the amount used here. The fact that these rabbits withstood such a large injection without showing any evident disturbance seems to indicate that they had a specific immunity.

A pig weighing 30 pounds was fed 100 cc. of one of the cultures received from Dr. Hirschfeld mixed with its food. For the next 4 days its temperature was increased and it ate very little. Its feces were diarrheal in character but the organism fed was not obtained on Endo plates made from the feces the 5th day after the feeding. On this day its temperature approached normal, it was eating well and appeared to be much livelier than on the days following the feeding. On the 5th day after the feeding virulent hog-cholera virus was injected intramuscularly, as it has been found in similar experiments that hog-cholera bacilli introduced into the digestive tract by feeding will act as secondary invaders when the pig is infected with virus. The pig died 8 days after the injection of the virus and was autopsied soon after death. It showed lesions characteristic of hog-cholera with the addition of a grayish, membranous necrosis of the mucosa of the large intestine. Plate cultures were made from the spleen, two mesenteric lymph nodes, the exudate in the colon, the mucous membrane of the colon after removing the exudate, the kidney, and the liver. In all, thirty-five subcultures from these plates were studied. They resembled the organism fed, with the following exceptions. Of six cultures from the mucosa of the colon, one failed to form hydrogen sulfide or ferment dulcite, but acted on arabinose. The other cultures were the same as the culture fed. Of two cultures from the kidney, one was the same as the organism fed, while the other differed in that it failed to act on dulcite. It did, however, produce hydrogen sulfide and on the second test fermented dulcite promptly. Only two cultures from the liver were examined and neither acted on dulcite, while one did and the other did not ferment

arabinose. Both formed hydrogen sulfide and on the second test both fermented dulcite and arabinose. The culture from the colon is, then, the only one that has shown any permanent change, and this culture 2 months after its isolation still failed to form hydrogen sulfide or ferment dulcite. It has, then, approached the hog-cholera bacillus in its cultural characters. The organisms from the liver and kidney also approached the hog-cholera bacillus in cultural character, but they soon regained the properties lost. The question as to whether the cultures recovered are the ones fed cannot positively be decided. Seven other pigs of the same litter have been infected with hog-cholera virus and bacteriological examination has failed to show the hog-cholera bacillus, though from some of them swine-typhus bacilli have been isolated. All the cultures from this pig resembled the hog-cholera bacillus serologically so the probabilities are that they were the descendants of organisms fed.

One similar test has been made with a swine-typhus culture and in this one case the feeding failed to cause a rise in temperature. Cultures made at autopsy, the animal having been infected with hog-cholera virus, failed to show swine-typhus bacilli.

When hog-cholera bacilli are fed, the pig reacts as did the animal fed the Paratyphoid C culture. There is an increased temperature beginning the day after the feeding and lasting for from 3 to 4 days. If at the end of this time hog-cholera virus is injected into the pig, hog-cholera bacilli will be found in the organs at autopsy.

DISCUSSION.

While these organisms isolated by Hirschfeld (1) are not typical hog-cholera bacilli in that they ferment dulcite and arabinose, produce hydrogen sulfide, and are not virulent for rabbits, their serum reactions are so characteristic that they should be placed in the hog-cholera bacillus group. These serum reactions are very fundamental, much more so than are the fermentations of the rarer carbohydrates.

There are several possible explanations which might account for these differences. One is that in the region from which these organisms were obtained atypical strains of hog-cholera bacilli exist in swine. Another is that the organisms in swine may be typical but

after passing to man they have become modified. A third possibility is that these organisms did not come from swine. They do not, however, correspond to any of the animal paratyphoids that have been described. What appear to be culturally typical hog-cholera bacilli do exist in nearby regions, as is shown by the observations of Trawinski (8). He isolated forty-two cultures from swine imported into Germany from Poland. All the cultures failed to ferment dulcite and arabinose, while three cultures of so called *Bacillus suipestifer* obtained from Kral's collection acted on these carbohydrates. One so called *suipestifer* strain obtained from Budapest acted the same as the cultures he isolated and was agglutinated to the titer limit by serum of an animal immune to one of his strains. The cultures from Kral were not agglutinated to the titer limit by this serum. He does not record the virulence of the cultures for rabbits or the production of hydrogen sulfide, but notes that all of his forty-two cultures formed acid but no gas in sorbite, whereas the control cultures of *Bacillus suipestifer* formed gas.

It is an interesting fact that the hog-cholera bacillus, which at one time was so commonly present in swine infected with hog-cholera that it was regarded as the cause of the disease, has not been found more frequently in man. In the older literature of food poisonings some of the organisms isolated were virulent when injected subcutaneously into rabbits and in some cases necroses were found in the livers of these animals. These facts indicate that the hog-cholera bacillus may have been the organism that was being studied, but the evidence is not conclusive.

Reed and Carroll (9) made a comparative study of *Bacillus icteroides* (Sanarelli) and the hog-cholera bacillus and concluded that they were the same. In cultural characters, virulence, and the disease produced in animals the two cultures were identical. They made only a few agglutination tests and the results are not very clear-cut but they indicate a relation between the two organisms. We are fortunate in having in our collection a culture of *Bacillus icteroides* that was received directly from Sanarelli and it is agglutinated to the titer limit in anti-hog-cholera bacillus serum and absorbs the agglutinins from this serum. In addition it fails to ferment dulcite and arabinose and does not produce hydrogen sulfide, thus resembling the hog-

cholera bacillus, while it differs from the latter organism in that it is not virulent for rabbits.

There is another culture in the collection labeled paratyphoid B Longcope, which culturally and serologically is a hog-cholera bacillus but which is not virulent for rabbits. The chances are that this culture came from a case of paratyphoid reported by Longcope (10) in 1902, but we cannot be sure of this fact. The probabilities are that it is at least of human origin.

As far as I know these are the only cultures from man that correspond closely to the hog-cholera bacillus so that an outbreak in the Balkan region with which organisms of the hog-cholera bacillus group are associated is of great interest. There must be many opportunities for hog-cholera bacilli to infect man, but they either rarely find conditions such that they can grow in the human body or, what is less likely, they do grow and quickly lose their distinguishing characters.

CONCLUSIONS.

1. The organisms isolated by Hirschfeld from febrile cases resembling paratyphoid fever and named Paratyphoid C can be placed in the hog-cholera bacillus group by their agglutination absorption properties though they are not typical culturally.

2. When fed to a pig a febrile disease resulted from which the animal recovered. After injection of hog-cholera virus the organisms fed were found generally distributed and some of them had lost cultural characters so that they are brought into the class of typical hog-cholera bacilli except for their low virulence for rabbits.

3. While hog-cholera bacilli have many opportunities to infect man they either are not able to grow in the human body or, what is less likely, they do grow and lose the characters that distinguish them.

The writer is indebted to Dr. L. Hirschfeld, Director of the Laboratory Service of the Serbian Army, for the cultures, to Professor David Klein, of the School of Hygiene, the Johns Hopkins University, for bringing them to this country, and to Mr. Henry Hagens, of this Laboratory, for technical assistance.

BIBLIOGRAPHY.

1. Hirschfeld, L., *Lancet*, 1919, i, 296.
2. MacAdam, W., *Lancet*, 1919, ii, 189.
3. Mackie, F. P., and Bowen, G. J., *J. Roy. Army Med. Corps*, 1919, xxxiii, 154.
4. Schütze, H., *Lancet*, 1920, i, 93.
5. Jordan, E. O., *J. Infect. Dis.*, 1917, xx, 457.
6. Krumwiede, C., Jr., Kohn, L. A., and Valentine, E., *J. Med. Research*, 1918, xxxviii, 89.
7. TenBroeck, C., *J. Exp. Med.*, 1920, xxxii, 19.
8. Trawinski, A., *Centr. Bakteriol., 1te Abt., Orig.*, 1918, lxxx, 339.
9. Reed, W., and Carroll, J., *J. Exp. Med.*, 1900, v, 215.
10. Longcope, W. T., *Am. J. Med. Sc.*, 1902, cxxiv, 209.

STUDIES ON CALCIUM AND MAGNESIUM METABOLISM IN DISEASE.

I. Calcium and Magnesium Metabolism in Leprosy.

By FRANK P. UNDERHILL, Ph. D., JAMES A. HONEIJ, M.D., and L. JEAN BOGERT, Ph.D.

With the Cooperation of Myrtle L. Aldrich.

(*From the Laboratories of Experimental Medicine and Radiology of the School of Medicine, Yale University, New Haven.*)

(Received for publication, February 16, 1920.)

In a preliminary paper by Honeij[1] on bone changes in leprosy it was shown that in this disease there is a definite absorption of bone salts. The idea seems prevalent that the bony changes in leprosy are due to suppurative processes or to nerve lesions, and hence little attention has been paid to them. This view-point is, however, entirely erroneous, for although bony changes may bear a relation to suppurative processes and to nerve lesions, Honeij points out that this kind of reaction plays no part in the true type of bone absorption which is part of the general disease. Thus, in the cases of leprosy with absence of suppuration and nerve disturbances the absorption of bone salts is demonstrable. Again, in progressive cases without any marked evidence of nerve involvement and distinctly without indication of suppuration the absorption of bone salts and consequent changes are most noticeable.

From the foregoing considerations it is apparent that a study of the calcium and magnesium exchange in leprosy would possibly afford a better conception of the processes involved in the abnormal condition under discussion. Indeed, the possibility exists that such a study might lead to improvement in treatment or prognosis as well as be of importance in the classification of the various types of leprosy.

[1] Honeij, J. A., *Am. J. Roentgenol.*, 1917, iv, n. s., 494.

Plan of Investigation.

Experience has demonstrated that simple determination of the balance of calcium and magnesium in the body does not always lead to convincing conclusions. This is due in large measure to the fact that it is difficult to obtain approximately perfect balances, and even though attained this maintenance is not easy. An attempt to draw definite conclusions from the balances alone is even more difficult in diseased conditions wherein the elements concerned exert a disturbing influence. Therefore in the present investigation, although due weight has been given to the calcium and magnesium balances, more significance has been attached to giving the diseased organism a distinct task to perform and observing the manner in which this is accomplished. Stated differently, the general plan of procedure has been to maintain diseased individuals upon a restricted diet low in calcium and magnesium and then for a period to increase the calcium or magnesium either by administration of food rich in the element or directly as a salt. From the data so obtained various ratios were calculated. A comparison was then made with the ratios obtained from normal individuals maintained under similar experimental conditions. In this manner deviations from normal behavior are readily detected, inasmuch as usually, when perverted calcium or magnesium exchange occurs, the evidence is quite noticeable.

Method.—The dietary was limited in the kind of food, and, as has been stated previously, was so selected as to be poor in calcium and magnesium quantitatively. The dietary was restricted within certain limits but was not a constant diet. Calcium was added to this diet in the form of milk, magnesium as magnesium citrate. The investigation was divided into four periods. Previous to the first period all subjects were maintained upon the diet of Period 1 for 3 days. Period 1 was arranged so as to represent a calcium-magnesium-low interval. Period 2 represented a calcium-rich but magnesium-low period. This was accomplished by the addition of varying quantities of milk. Period 3 was approximately identical with Period 1; that is, a period low in both calcium and magnesium, milk being withdrawn from the dietary. Period 4 was essentially the same as Period 3 except that 2 gm. of magnesium citrate, equivalent to 205 mg. of magnesium, were admin-

istered in three equal doses daily. In other words, Period 4 was an interval of low calcium and high magnesium.

The experimental periods were marked off in the feces by the employment of carmine. Calcium and magnesium were determined in the food, urine, and feces by the methods of McCrudden.[2]

Description of Subjects.

Normal Individuals.

Subject 1.—M. A., female, age 25 years; engaged in laboratory work. The experiment was so planned that menstrual periods were avoided.

Subject 2.—G. S., male, age 28 years; engaged in laboratory work.

Leprous Cases.

Case 1.—M. T., male, age 31 years; single; laborer; born in Greece. December 1, 1916. Admitted to the New Haven Hospital.

Complaint.—"Red spots on body." A few spots first appeared on the face, resembling lesion now present. These lesions were at first small and red, later became browner and larger. Cheeks were at first affected, then the feet, following these the arms and hands. Lesions are not painful, have never itched, never broken down to form ulcers. Patient claims he feels well. For less than a year the left side of the nose has been almost completely blocked; no catarrh previously.

Family History.—Negative.

Past History.—Negative.

Physical Examination.—Negative except in the following respects. The nose shows thickening of the septum on the left side, with whitish color and several small whitish nodules. Glands of the cervical, axillary, epitrochlear, and inguinal regions are all moderately enlarged. The skin shows the greatest changes. On the face and forehead rather numerous, flat papules 2 to 4 mm. in size and of a brownish color. An infiltrated area 1 cm. in diameter seen on right cheek and two similar areas on left cheek. Pigmented areas also seen. A nodule 1.5 cm. in diameter on left forehead, involving skin and subcutaneous tissue. The same lesions are found on forearm, elbows, buttocks, thighs, and lower legs, mostly on extensor surfaces. The nerves are thickened; the ulnar nerves, peroneal, and popliteal are also affected. Many large papules and a large area on left shin are anesthetic. Many pigmented areas are slightly affected. The blood examination on November 8, 1917, shows 63 per cent hemoglobin and 8.4 per cent white cells, mostly polymorphonuclears. Wassermann reaction +++. Later the white cell blood count was 11,000 with 75 per cent polymorphonuclears. Achroma also.

---

[2] McCrudden, F. M., *J. Biol. Chem.*, 1909–10, vii, 83; 1911–12, x, 187.

Renal functional test, December 18, 1917, shows 54 per cent excretion 2 hours after phenolsulfonephthalein. The urine examination gave a specific gravity of 1,015; the color was amber; clear; acid reaction; slight possible trace of albumin; no sugar; few epithelial cells on microscopic examination.

The radiographic gastrointestinal examination was negative except for some colonic stasis.

The patient was under treatment for 1 year and on special diet from June 26 to July 25, 1917.

Diagnosis.—Leprosy of the nodular, anesthetic type.

Case 2.—G. C., male, age 21 years; single; laborer; born in Italy. January 2, 1918. Admitted to the New Haven Hospital.

Complaint.—Difficulty in breathing through nose. Patient has been troubled with a dry feeling in his nose for the last 2 years. Has expelled by force scab-like masses which are now and then accompanied by slight epistaxis. Has always had a biting sensation in his nose. Patient is not subject to colds. Throat troubles him also. Headaches are frequent and quite frequently has cold sweats. Every now and then his upper extremity "falls asleep," giving tingling sensation. Itchy feeling when body becomes warm.

Family History.—Negative.

Past History.—Negative.

Physical Examination.—Negative except in the following respects. The nose shows both anterior nares almost entirely occluded by atrophic scabs. On removing them a perforation of the septum is seen 3 by 2 cm.; it does not seem to include the bony portion of the septum. The turbinates are difficult to determine. The eyes show a slight conjunctivitis and areas of anesthesia which also occur on lids. A small nodule is seen at the sclera-corneal margin of both eyes. The glands of the cervical, axillary, and inguinal regions are enlarged. The examination of the body is negative except for areas of anesthesia, fairly symmetrical in their distribution, occurring on body and on extremities, face, and ears. The hands and feet are cyanotic. The patient perspires freely. The nerves are thickened. The ulnar and anterior tibial nerves are considerably thickened. Areas of discoloration and pigmentation seen on the buttocks; here there are also some nodules. The blood examination on October 16, 1918, shows a white blood count of 8,400 and on October 21 a count of 13,600. The urine examination on October 15 gave a specific gravity of 1,032; normal color; clear; acid reaction. Microscopic examination showed a rare leucocyte.

Diagnosis.—Leprosy of the anesthetic type. An earlier case of the disease than Case 1.

Dietary.

The diet for all subjects consisted of varying but definite quantities of the foodstuffs presented in Table I. The calcium content and magnesium content are also given. In order to save space the quantities of food consumed daily are omitted.

TABLE I.

Dietary.

Food.	Calcium content.	Magnesium content.
	per cent	*per cent*
Bread	0.032	0.036
Orange	0.056	0.015
Egg	0.073	0.015
Sugar		
Baked potato	0.005	0.020
Chopped beef	0.007	0.018
Banana	0.013	0.021
Prunes	0.042	0.018
Boiled rice	0.002	0.005
Fresh strawberries	0.041	0.019
Milk	0.179 0.199	0.016 0.014

Calcium and Magnesium Balances.

In Tables II to V are presented the data relative to the calcium and magnesium exchange in the four subjects of investigation. The first period shows the two normal subjects to be in a distinct negative calcium balance, but on the other hand, the magnesium balance is positive. Added calcium, in the form of milk, in the second period causes both subjects to approach an equilibrium. In Subject 1 the added calcium exerted little or no influence upon the magnesium balance, whereas with the other normal individual, Subject 2, a distinct negative magnesium balance was exhibited. Withdrawal of the additional calcium in the third period resulted with the two normal subjects in a distinct negative calcium balance; the negative magnesium balance in this period was not so marked. In the fourth period where magnesium citrate was added to the diet a significant

TABLE II.

Subject 1; Normal. Calcium and Magnesium Balances.

Period.	Diet.	Date.	Calcium.				Magnesium.			
			Intake.	Output.		Balance.	Intake.	Output.		Balance.
				Urine.	Feces.			Urine.	Feces.	
		1919	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	Calcium-low and magnesium-low.	Jan. 4	317	316			228	67		
		" 5	349	300			239	93		
		" 6	333	335			263	96		
		" 7	342	395			275	88		
Total			1,341	1,346	885	−890	1,005	344	585	+76
				2,231				929		
Average per day			335	336	221	−222	251	86	146	+19
				557				232		
2	Calcium-high and magnesium-low.	Jan. 8	1,324	469			341	109		
		" 9	1,333	508			361	110		
		" 10	1,339	618			392	134		
Total			3,996	1,595	2,500	−99	1,094	353	676	+65
				4,095				1,029		
Average per day			1,332	532	833	−33	364	117	225	+22
				1,365				343		
3	Calcium-low and magnesium-low.	Jan. 11	365	325			294	108		
		" 12	329	352			277	124		
		" 13	358	343			271	114		
Total			1,052	1,020	881	−849	842	346	691	−195
				1,901				1,037		
Average per day			351	340	293	−283	280	115	230	−65
				633				345		
4	Calcium-low and magnesium-high.	Jan. 14	367	351			487	128		
		" 15	419	426			507	152		
		" 16	408	408			533	148		
Total			1,194	1,185	1,031	−1,022	1,527	428	710	+389
				2,216				1,138		
Average per day			398	395	343	−341	509	142	236	+130
				738				379		

TABLE III.

Subject 2; Normal. Calcium and Magnesium Balances.

Period.	Diet.	Date.	Calcium. Intake.	Calcium. Output. Urine.	Calcium. Output. Feces.	Calcium. Balance.	Magnesium. Intake.	Magnesium. Output. Urine.	Magnesium. Output. Feces.	Magnesium. Balance.
		1919	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
1	Calcium-low and magnesium-low.	Jan. 4	506	149			345	128		
		" 5	542	215			398	124		
		" 6	537	213			369	112		
		" 7	584	242			429	104		
		" 8	525	359			377	161		
Total			2,694	1,178	2,264	−748	1,918	629	1,257	+32
				3,442				1,886		
Average per day			539	235	452	−150	383	125	251	+6
				688				377		
2	Calcium-high and magnesium-low.	Jan. 9	1,539	295			494	176		
		" 10	1,467	304			450	183		
		" 11	1,499	400			489	165		
Total			4,505	999	3,479	+27	1,433	524	1,132	−223
				4,478				1,656		
Average per day			1,501	333	1,159	+9	477	174	377	−74
				1,492				552		
3	Calcium-low and magnesium-low.	Jan. 12	444	207			363	134		
		" 13	502	215			382	145		
		" 14	537	415			417	110		
Total			1,483	837	1,735	−1,089	1,162	389	822	−49
				2,572				1,211		
Average per day			494	279	578	−363	387	129	274	−16
				857				403		
4	Calcium-low and magnesium-high.	Jan. 15	544	280			612	185		
		" 16	472	382			560	169		
		" 17	592	772			595	101		
Total			1,608	1,434	1,390	−1,216	1,767	455	976	+336
				2,824				1,431		
Average per day			536	478	463	−405	589	151	325	+112
				941				477		

TABLE IV.

Case 1; Leprosy. Calcium and Magnesium Balances.

Period.	Diet.	Date.	Calcium. Intake.	Calcium. Output. Urine.	Calcium. Output. Feces.	Calcium. Balance.	Magnesium. Intake.	Magnesium. Output. Urine.	Magnesium. Output. Feces.	Magnesium. Balance.
		1917	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	Calcium-low and magnesium-low.	June 30	290	22			194	60		
		July 1	217	21			174	77		
		" 2	277	23			198	77		
		" 3	299	23			184	69		
Total			1,083	89	560	+434	750	283	223	+244
				649				506		
Average per day			271	22	140	+108	187	71	56	+61
				162				126		
2	Calcium-high and magnesium-low.	July 4	894	23			204	89		
		" 5	1,005	20			214	81		
		" 6	976	25			226	100		
Total			2,875	68	1,083	+1,724	644	270	230	+144
				1,151				500		
Average per day			958	22	361	+574	214	90	76	+48
				383				166		
3	Calcium-low and magnesium-low.	July 7	203	12			126	63		
		" 8	265	28			171	75		
		" 9	177	25			127	77		
Total			645	65	931	−351	424	215	299	−90
				996				514		
Average per day			215	22	310	−117	141	71	99	−30
				332				171		
4	Calcium-low and magnesium-high.	July 10	286	27			408	94		
		" 11	253	27			362	95		
		" 12	257	28			395	92		
Total			796	82	761	−47	1,165	281	490	+394
				843				771		
Average per day			265	27	253	−15	388	93	163	+131
				281				257		

TABLE V.

Case 2; Leprosy. Calcium and Magnesium Balances.

Period.	Diet.	Date.	Calcium.				Magnesium.			
			Intake.	Output.		Balance.	Intake.	Output.		Balance.
				Urine.	Feces.			Urine.	Feces.	
		1917	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	Calcium-low and magnesium-low.	Feb. 10	523	103			324	114		
		" 11	522	129			344	139		
		" 12	486	153			339	106		
		" 13	496	119			331	87		
Total			2,027	504	1,538	−15	1,338	446	994	−102
				2,042				1,440		
Average per day			506	126	384	−4	334	111	248	−26
				510				360		
2	Calcium-high and magnesium-low.	Feb. 14	1,519	207			381	122		
		" 15	1,466	179			362	126		
		" 16	1,929	189			448	126		
Total			4,914	575	2,944	+395	1,191	374	981	−164
				3,519				1,355		
Average per day			1,638	192	981	+131	397	124	327	−54
				1,173				451		
3	Calcium-low and magnesium-low.	Feb. 17	492	135			339	109		
		" 18	517	159			353	124		
		" 19	515	161			379	115		
Total			1,524	455	1,169	−100	1,071	348	852	−129
				1,624				1,200		
Average per day			508	151	389	−33	357	116	284	−43
				541				400		
4	Calcium-low and magnesium high.	Feb. 20	562	145			592	144		
		" 21	514	152			580	148		
		" 22	525	138			542	151		
Total			1,601	435	1,137	+29	1,714	443	1,122	+149
				1,572				1,565		
Average per day			533	145	379	+9	571	147	374	+49
				524				521		

negative calcium balance persisted, but the magnesium balance became positive.

It is therefore evident that with normal subjects in negative calcium balance added calcium causes the balance to become positive and that the additional calcium exerts little or no influence upon the magnesium balance. The influence of the added calcium is, however, only temporary, since its withdrawal results in a resumption of a significant negative calcium balance. Added magnesium changes a slightly negative magnesium balance to a strongly positive one without a very significant influence upon the calcium balance.

With the leprosy cases a different picture is presented. Although both subjects were maintained on a low calcium intake in Period 1, Case 1 was in positive calcium balance and Case 2 was almost in perfect equilibrium. Case 1 was also in positive magnesium balance, whereas Case 2 showed a negative balance. Administration of added calcium in Period 2 demonstrated in both instances retention of calcium, greater in Case 1 than in Case 2. With Case 1 the magnesium balance was little altered, whereas with Case 2 the balance was more strongly negative than it was in Period 1. In Period 3, during which the additional calcium was eliminated, both calcium and magnesium were negative with respect to equilibrium. In the fourth period added magnesium caused a positive magnesium balance, and with Case 2 a positive calcium balance was also obtained. With Case 1 an approach to calcium equilibrium was evident.

A comparison of these results with those of the normal subjects shows that there is an apparent tendency for the leprosy subjects to remain in positive balance with respect to calcium, and in Case 1 in magnesium balance also. It is also evident that this tendency is, with respect to calcium, greater in the more advanced case, No. 1, than in the other patient. It is therefore indicated that in leprosy the organism exhibits a tendency to retain calcium whether maintained upon a diet containing little or much of this element, the more advanced the pathological condition clinically the more evident does this tendency to retention become.

In leprosy the organism may or may not retain a large portion of the added magnesium. Where retention was in evidence its degree was not strikingly greater than in the normal individuals maintained under similar conditions.

Further Analysis of the Data.

Calcium Metabolism.

In order to ascertain the correctness of the statement that in leprosy there is a tendency to retain calcium, the data have been subjected to further analysis.

Excretion of Added Calcium.—If one calculates the percentage of added calcium eliminated from the body during the period of administration, *i.e.* Period 2, it will be found that in leprosy the figures are different from those for normal individuals (Table VI).

TABLE VI.

Excretion of Added Calcium.

Subject.	Excretion of added calcium.*
	per cent
Subject 1; normal	100+
" 2; "	92+
Case 1; leper	38+
" 2; "	62+

* Period 2; calcium added as milk.

From these figures it is apparent that normal individuals in negative calcium balance promptly eliminate added calcium almost quantitatively, only a small amount of added calcium being retained; this leads to a corresponding approach to calcium equilibrium. In leprosy added calcium is retained in large measure. In the two individuals studied a marked difference was observed in the ability of the body to retain calcium. The query is pertinent whether this difference in behavior is due to a difference in the stage of the disease. Clinically the two cases are distinct, Case 1 being in a more advanced stage than Case 2. From the fact that the more advanced case shows the greater retention of calcium it may be argued that this subject has the greater need for this element.

Relation of Calcium Output to Intake.—From an inspection of Table VII it is apparent that in leprosy calcium exchange is different from that found in normal individuals. Placed upon a low calcium

diet the leprous subjects exhibited a distinct tendency to retain calcium, the retention being especially marked in Case 1, the more advanced case. When placed upon a calcium-rich diet Case 1 retained enormous quantities of calcium, the other subject, Case 2, exhibiting this behavior to a much less marked degree. When again placed upon a calcium-poor diet both subjects tended to approach the normal individuals in their behavior. On the other hand, the

TABLE VII.

Relation of Calcium Output to Intake.

$$\textit{Ratio: } \frac{\textit{Calcium Output}}{\textit{Calcium Intake}}.$$

Subject.	Period.			
	1	2	3	4
Subject 1; normal	1.6	1.0	1.8	1.9
" 2; "	1.2	0.9	1.7	1.7
Case 1; leper	0.6	0.4	1.5	1.0
" 2; "	1.0	0.7	1.0	0.9

excretion was less marked again in the fourth period, and evidence of a tendency to retention is to be observed. This approach to a normal type of excretion may perhaps be interpreted to mean that the leprous organism once having a sufficiency of calcium for its present needs rather slowly parts with the excess.

Relation of Output to Intake for the Entire Period.—Comparison (Table VIII) of the total output in relation to intake for the entire

TABLE VIII.

Relation of Calcium Output to Intake for the Entire Period.

Subject.	Calcium.		
	Output.	Intake.	Difference.
	mg.	*mg.*	*mg.*
Subject 1; normal	10,443	7,583	−2,860
" 2; "	12,626 (13 days).	9,752	−2,874
Case 1; leper	3,639	5,399	+1,760
" 2; "	8,757	10,066	+1,309

period of investigation in the four subjects brings out the fact already alluded to; namely, a significant calcium retention in leprosy, even though the subjects were maintained for three periods out of four on a calcium-low diet. Again the fact is emphasized that in the more advanced patient, Case 1, the retention is greater than in the less advanced patient, Case 2. The normal subjects, on the other hand, exhibited a marked loss of calcium.

Relation of Calcium Intake and Output to Body Weight.—The weights of the individuals under experimentation varied widely and it is possible that this variation may bear a relation to the interpretation of the facts discussed above. With this idea in mind the relation of the total intake and output of calcium to body weight

TABLE IX.

Relation of Calcium Intake and Output to Body Weight.

Subject.	Body weight.	Calcium.		Calcium per pound of body weight.	
		Intake.	Output.	Intake.	Output.
	lbs.	*mg.*	*mg.*	*mg.*	*mg.*
Subject 1; normal	105	7,583	10,443	72	99
" 2; "	150 (13 days).	9,752	12,626	65	84
Case 1; leper	117	5,399	3,639	46	31
" 2; "	147	10,066	8,757	68	59

has been expressed numerically in Table IX. Inspection of these data will show at once the same general type of differences already discussed, so that it may be concluded that variations in body weight do not account for the fact that in leprosy calcium is used differently than in the normal organism.

Absorption of Calcium.—A point of considerable interest in the interpretation of the data presented relates to the question of variation in absorption of the introduced calcium. It is obvious that this question does not lend itself readily to dogmatic statements, since it is probable that calcium is excreted in variable degree by both the kidney and intestine under diverse circumstances. However, in the present discussion it is pertinent to compare the absorption of calcium in leprosy with that of normal individuals. In this

consideration the excretion of calcium through the urine is taken tentatively as evidence of absorption; the passage of calcium from the body through the feces as unabsorbed calcium. This is obviously incorrect, since undoubtedly part of the calcium eliminated with the feces is calcium which underwent absorption and is merely excreted by the intestine. For a comparative study of absorption, however, such a hypothesis may be employed.

On the basis of this hypothesis, with the necessary limitations involved, a study of the excretion of calcium by way of the urine in relation to the intake shows some noteworthy features (Table X). With the normal individuals on a calcium-low diet from approximately one-half to a quantity equal to the ingested calcium is excreted

TABLE X.

Relation of Urinary Calcium Excretion to Calcium Intake.

$$\textit{Ratio: } \frac{\textit{Calcium of Urine}}{\textit{Calcium of Food}}.$$

Subject.	Period.			
	1	2	3	4
Subject 1; normal	1.0	0.4	1.0	1.0
" 2; "	0.4	0.2	0.5	0.8
Case 1; leper	0.08	0.02	0.1	0.1
" 2; "	0.2	0.1	0.3	0.2

by way of the kidney. As a calcium-rich diet is introduced the relative quantity of food calcium absorbed diminishes to a perceptible degree. Withdrawal of the calcium-rich diet causes the relation between the calcium excreted by the kidney and that ingested to resume the status which obtained in the calcium-poor period previously.

With the cases of leprosy the ratio is radically different from that of the normal individuals. In both cases relatively little calcium is eliminated by way of the urine. This is especially noticeable with the more advanced patient, Case 1. On a calcium-rich diet both subjects follow the general type of behavior of normal subjects; the degree of deviation is markedly different. The same fact holds when the calcium-poor diet is again introduced.

Relation of Fecal Calcium to Calcium Intake.—The figures given, when applied to the hypothesis outlined above, would lead one to conclude that very much less calcium was absorbed in the leprous cases than in the normal individuals. However, before positive conclusions can be drawn relative to this point a study should be made of the relation of calcium excretion by way of the feces to the calcium intake. This has been done and the results are expressed in Table XI. These figures demonstrate that normal conditions exist with respect to calcium elimination by the intestine in leprosy when these subjects are placed upon a calcium-poor diet. Addition of calcium to the diet results in a relatively smaller quantity

TABLE XI.

Relation of Fecal Calcium to Calcium Intake.

$$\textit{Ratio: } \frac{\textit{Calcium of Feces}}{\textit{Calcium of Food}}.$$

Subject.	Period.			
	1	2	3	4
Subject 1; normal	0.6	0.6	0.8	0.8
" 2; "	0.8	0.7	1.1	0.8
Case 1; leper	0.5	0.3	1.4	0.9
" 2; "	0.7	0.6	0.7	0.7

appearing in the feces during this period. Since in this period there is no increase in either the urinary or fecal excretion of calcium it is obvious that the added calcium was absorbed in large measure. This theory is supported by the fact that in the later periods upon a calcium-poor diet some of the retained calcium is excreted by both the urine and feces (see Periods 3 and 4, Tables X and XI). The conclusion is therefore warranted that the behavior of leprous subjects with respect to urinary calcium excretion cannot be ascribed to lack of absorption of this element. Moreover, absorption of calcium in the leprous condition seems even better than in the normal individual. This is especially true with the more advanced patient, Case 1.

Relation of Urinary Calcium to Fecal Calcium.—If a comparison is made of the output of calcium in the urine and feces it becomes evident (Table XII) that only a small proportion of absorbed calcium is excreted in the more advanced case of leprosy. With the less advanced case the behavior is more nearly normal.

TABLE XII.

Relation of Urinary and Fecal Calcium.

Ratio: $\frac{\textit{Calcium of Urine}}{\textit{Calcium of Feces}}$.

Subject.	Period.			
	1	2	3	4
Subject 1; normal	1.5	0.6	1.1	1.1
" 2; "	0.5	0.2	0.4	1.0
Case 1; leper	0.1	0.06	0.07	0.1
" 2; "	0.3	0.2	0.4	0.3

When compared to normal individuals under the same experimental conditions leprous patients retain calcium in relatively large quantities whether the individual is maintained upon a calcium-poor or a calcium-rich diet, and the greater the intake the greater is the relative retention. In the more advanced stage of the disease the degree of retention is greater than in the early phase. This retention cannot be explained by differences of calcium intake, or by variation in the power of absorption. It is apparently an evidence of metabolic demand for calcium induced by the disease. Clinically, it may be inferred that there is a need for calcium; experimentally, the organism gives demonstration of this need by retention of calcium administered.

It is perhaps self-evident that the loss of bone salts in leprosy is but a manifestation of the disease processes. Nevertheless, it is conceivable that under dietary conditions in which calcium is not particularly abundant the lack of this element may play a material part in the rapidity of the progress of the disease. Conversely, the suggestion is pertinent that the progress of the disease may be greatly retarded or perhaps even alleviated if an abundance of calcium is

present in the diet. The suggestion that plenty of calcium should be supplied in the food as a therapeutic measure is at least worthy of trial.

Magnesium Metabolism.

Excretion of Added Magnesium.—From the figures in Table XIII it is apparent that normal individuals in a slightly negative magnesium balance retain more than one-half the magnesium added to a diet relatively low in this element. Of the leprosy patients, Case 1 behaved in a manner very similar to the normal individuals, whereas in the less advanced patient, Case 2, three-quarters of the administered magnesium was promptly eliminated.

TABLE XIII.

Excretion of Added Magnesium.

Subject.	Excretion of added magnesium.
	per cent
Subject 1; normal	36
" 2; "	45
Case 1; leper	36
" 2; "	75

Relation of Magnesium Output to Intake.—As may be seen from an inspection of Table XIV leprous individuals differ little from normal individuals in the manner in which magnesium is eliminated from the body, whether these subjects are maintained upon a diet low or rich in magnesium.

TABLE XIV.

Relation of Magnesium Output to Intake.

$$\textit{Ratio:}\ \frac{\textit{Magnesium Output}}{\textit{Magnesium Intake}}.$$

Subject.	Period.			
	1	2	3	4
Subject 1; normal	0.9	0.9	1.2	0.7
" 2; "	0.9	1.1	1.0	0.8
Case 1; leper	0.6	0.8	1.2	0.7
" 2; "	1.0	1.1	1.1	0.9

Relation of Output to Intake for the Entire Period.—It is only when comparison is made between the total output and total intake of magnesium that a marked difference can be observed between normal individuals and leprous subjects. From Table XV it is seen that in spite of the fact that Case 1 ingested only about one-half or less of the magnesium taken in by the normal subjects, yet the absolute quantity of magnesium retained was more than double that of the normal subject retaining the most. It would appear, therefore, that in this case there is indication of a distinct magnesium retention. This would naturally be expected, since clinically in this patient there was evidence of considerable bone absorption. In Case 2, however, evidence of bone absorption was less apparent. With this

TABLE XV.

Relation of Magnesium Output to Intake for the Entire Period.

Subject.	Magnesium.		
	Output.	Intake.	Difference.
	mg.	*mg.*	*mg.*
Subject 1; normal	4,133	4,468	+335
" 2; " (13 days)	5,806	5,894	−88
Case 1; leper	2,291	2,983	+692
" 2; "	5,560	5,314	−246

subject there is a small loss of magnesium rather than a retention. It is therefore probable that in the advanced stages of leprosy the organism conserves its magnesium store, since it retains a considerable quantity. Although the advanced case of leprosy retained about the same percentage of food magnesium, the absolute amount retained was much greater, as may be seen from Table XV.

Relation of Magnesium Intake and Output to Body Weight.—When a comparison is made of the intake and output of magnesium to body weight it may be seen (Table XVI) that although the intake of the advanced leprous patient, Case 1, was much smaller than that of either the normal subjects or the other patient, nevertheless more magnesium was stored.

TABLE XVI.

Relation of Magnesium Intake and Output to Body Weight.

Subject.	Body weight.	Magnesium.		Magnesium per pound of body weight.	
		Intake.	Output.	Intake.	Output.
	lbs.	*mg.*	*mg.*	*mg.*	*mg.*
Subject 1; normal	105	4,468	4,133	42	39
" 2; "	150 (13 days).	5,894	5,806	39	38
Case 1; leper	117	2,983	2,291	25	19
" 2; "	147	5,314	5,560	36	38

Absorption of Magnesium.—From a study of Tables XVII and XVIII it is evident that the absorption of magnesium in leprosy falls within normal limits.

TABLE XVII.

Relation of Urinary Magnesium Excretion to Magnesium Intake.

$$\textit{Ratio: } \frac{\textit{Magnesium of Urine}}{\textit{Magnesium of Food}}.$$

Subject.	Period.			
	1	2	3	4
Subject 1; normal	0.3	0.3	0.4	0.2
" 2; "	0.3	0.3	0.3	0.2
Case 1; leper	0.3	0.4	0.5	0.2
" 2; "	0.3	0.3	0.3	0.2

TABLE XVIII.

Relation of Fecal Magnesium to Magnesium Intake.

$$\textit{Ratio: } \frac{\textit{Magnesium of Feces}}{\textit{Magnesium of Food}}.$$

Subject.	Period.			
	1	2	3	4
Subject 1; normal	0.5	0.6	0.8	0.4
" 2; "	0.6	0.8	0.7	0.5
Case 1; leper	0.3	0.3	0.7	0.4
" 2; "	0.7	0.8	0.8	0.6

Relation of Urinary Magnesium to Fecal Magnesium.—A comparison of the ratio of urinary magnesium to fecal magnesium demonstrates a marked difference in the case of advanced leprosy from that of the other subjects (Table XIX). In the normal individuals studied approximately one-third of the total magnesium excretion is by way of the urine. This relation holds fairly constant whether the individual is maintained on a low magnesium diet or whether additional magnesium has been administered. In general, with Case 1, on the other hand, almost as much or somewhat more magnesium is excreted by the urine than by the feces as long as the individual is on a magnesium-poor diet. When this element is added to the diet the ratio characteristic of the normal individual is obtained.

TABLE XIX.

Relation of Urinary and Fecal Magnesium.

$$\text{Ratio: } \frac{\text{Magnesium of Urine}}{\text{Magnesium of Feces}}.$$

Subject.	Period.			
	1	2	3	4
Subject 1; normal	0.6	0.5	0.5	0.6
" 2; "	0.5	0.4	0.4	0.4
Case 1; leper	1.2	1.2	0.7	0.6
" 2; "	0.4	0.4	0.4	0.4

A study of magnesium exchange in normal and leprous subjects demonstrates that in the advanced stage of leprosy there is a tendency for a retention of magnesium. This behavior is, however, not strikingly different from that of normal individuals maintained under similar dietary conditions.

Relation of Calcium to Magnesium.

Relation of Calcium and Magnesium in the Food.—In Table XX may be found the ratio of food calcium to food magnesium. Inspection of these data will demonstrate that in the first calcium-poor period, that is Period 1, the ratio is remarkably constant. In the second period, that in which calcium was added in the form of milk,

the ratio in the normal individuals changes markedly, but in the two instances is quite constant. With the leprosy subjects there is the same type of uniformity in the two individuals, but the ratio varies even further from that of Period 1 than in the normal subjects. With a return to a calcium-poor diet there is little variation in any subject from the figures for the first period. Added magnesium naturally lowers this ratio somewhat, but in no subject does it result in a perverted variation.

TABLE XX.

Relation of Calcium and Magnesium Intake.

$$\textit{Ratio: } \frac{\textit{Calcium of Food}}{\textit{Magnesium of Food}}.$$

Subject.	Period.			
	1	2	3	4
Subject 1; normal	1.3	3.6	1.2	0.8
" 2; "	1.4	3.1	1.2	0.9
Case 1; leper	1.4	4.3	1.5	0.7
" 2; "	1.5	4.1	1.4	0.9

Relation of Calcium and Magnesium in the Urine.—In the first calcium-poor period, in spite of the fact that the calcium-magnesium ratio in the food was fairly constant, there is a considerable variation in the ratio of urinary elimination of these substances even in normal individuals (Table XXI).

TABLE XXI.

Relation of Calcium and Magnesium in the Urine.

$$\textit{Ratio: } \frac{\textit{Calcium of Urine}}{\textit{Magnesium of Urine}}.$$

Subject.	Period.			
	1	2	3	4
Subject 1; normal	3.9	4.5	2.9	2.7
" 2; "	1.8	1.9	2.1	3.1
Case 1; leper	0.3	0.2	0.3	0.3
" 2; "	1.1	1.6	1.0	1.0

In the leprosy cases the ratio is distinctly perverted, especially in the advanced case. This perversion, however, may be explained by the retention of calcium by these subjects. When a calcium-rich diet is given the ratios vary more widely in the normal subjects than they do in the leprosy cases. Addition of magnesium does not alter the ratios in any determinable manner.

The most significant feature with respect to the ratio of calcium and magnesium in the urine is that a change from a calcium-poor diet to one rich in calcium, or the reverse, or a change from a magnesium-poor diet to a magnesium-rich diet does not induce any significant alteration in the unusual ratio of calcium to magnesium in the urine in the advanced case of leprosy, Case 1.

Relation of Calcium and Magnesium in the Feces.—As may be seen from inspection of Table XXII the relation between the calcium and

TABLE XXII.

Relation of Calcium and Magnesium in the Feces.

$$\textit{Ratio: } \frac{\textit{Calcium of Feces}}{\textit{Magnesium of Feces}}.$$

Subject.	Period.			
	1	2	3	4
Subject 1; normal	1.5	3.7	1.2	1.4
" 2; "	1.8	3.0	2.1	1.3
Case 1; leper	2.5	4.6	3.1	1.5
" 2; "	1.5	3.0	1.3	1.0

magnesium of the feces in leprosy does not differ widely from that in normal individuals. The greatest variation is in Case 1 and is caused by the fact that relatively less magnesium is eliminated through the feces than in the other subjects.

A study of the relations existing between the calcium and magnesium of the food, urine, and feces demonstrates that even in normal subjects, although the relation of food calcium and magnesium may be quite constant, nevertheless a decided variation may exist in the relation of these elements in the urine and feces, especially in the former. This fact is undoubtedly explained by the extent of retention of one or the other of these elements.

In leprosy the perversion of ratios is much more noticeable, especially in the advanced case. Inasmuch as relatively large quantities of calcium and much smaller quantities of magnesium were retained in the body it is evident that the greater retention of these elements in leprosy is sufficient to explain the peculiar ratios observed, for instance, in Table XXI.

CONCLUSIONS.

In leprosy there is a definite retention of calcium.

The more advanced the stage of the disease the greater is the degree of retention.

This behavior on the part of leprous individuals may be taken as an indication that the organism is in need of calcium.

Magnesium may also be retained in leprosy, but the degree of retention is much less marked than in the case of calcium.

With the exception of the retention of calcium and magnesium the leprous organism responds to changes in the intake of calcium and magnesium in the same manner as normal individuals.

The results of this investigation suggest that in leprosy administration of calcium may be of benefit as an additional therapeutic measure in an endeavor to retard or arrest the progress of the bone changes characteristic of the disease.

STUDIES ON CALCIUM AND MAGNESIUM METABOLISM IN DISEASE.

II. Calcium and Magnesium Metabolism in Multiple Cartilaginous Exostosis.

By FRANK P. UNDERHILL, Ph. D., JAMES A. HONEIJ, M.D., and L. JEAN BOGERT, Ph. D.

With the Cooperation of Myrtle L. Aldrich.

(From the Laboratories of Experimental Medicine and Radiology of the School of Medicine, Yale University, New Haven.)

(Received for publication, February 16, 1920.)

Clinically, the bone changes occurring in multiple exostosis stand in sharp contrast to those involved in leprosy. In leprosy bone salts are lost from the body; in multiple exostosis bone salts are deposited. In a previous paper[1] we have shown that in leprosy calcium is retained to a marked extent. The same tendency to retain magnesium is present but not to the same degree. In other words, the diseased organism conserves its bone-building constituents.

In the present communication observations upon two selected cases of multiple exostosis are presented. As far as we are aware there are no records of an extensive study of the calcium and magnesium exchange in this proliferative type of bone disease.

Plan of Investigation.

The general plan of the experiment and the methods and dietary employed were identical with those outlined in the previous paper.

Description of Subjects.

Normal Individuals.

The individuals (Subjects 1 and 2) serving as controls in the investigation on leprosy have been taken as controls in the present observations also.

[1] Underhill, F. P., Honeij, J. A., and Bogert, L. J., *J. Exp. Med.*, 1920, xxxii, 41.

Exostosis Cases.

Case 1.—R W. H., male, single; age 31 years; farmer and later clerk.

Complaint.—Pain in knees; tires easily; limitation of motion.

Family History.—No evidence of heredity from grandparents' generation.

Past History.—Has had usual childhood diseases; in 1912 had nervous breakdown with gastric disturbances; had gonorrhea in 1916; no syphilis.

Present History.—At 6 years of age father noticed an enlargement on the inside of right ankle. 2 years later similar enlargement of left ankle. No pain or difficulty in walking. Later, from time to time until 21 years of age "lumps" were discovered all over body especially under left arm, axilla, and on ribs. Between ages of 10 and 15 years lumps appeared on lower extremities and on left hip after an injury. At same time fell on coccyx and later a lump developed there. At age of 23 noticed a lump on outer side, right thigh; this apparently was a result of a fall 2 years previous; at that time there were distinct ecchymoses over that area. Certain of lumps have disappeared. One on wrist disappeared 5 years after first noticed, others under arm, on left wrist, and on thumb. Never had fractures.

Physical Examination.—The patient is disproportionate in size, although muscular, is in general poorly developed, is short of stature, pigeon-breasted, and with rather prominent bony angles. Patient walks much like an automaton, with short jerky steps. Weight 114 pounds. The lungs, heart, and other organs are negative.

Examination of the bony skeleton shows numerous growths, of different sizes and apparently of different consistency. Some of these growths are easily grasped and felt and others are barely recognized. They occur more or less symmetrically. Those most easily felt and even seen occur on the femur, near knee joint, tibia, fibula, radius, and ulna. Near the shoulder on the left is a very prominent growth. There is a marked curvature of the forearms. The radiographs demonstrate these growths to be bony with terminal cartilage crowns. Some smooth and regular, others markedly irregular and rough. A large number occur near the epiphysis; some are undoubtedly responsible for the limitation of motion. The bones themselves are well formed with rather prominent and thickened epiphyseal portions.

The urine and blood examinations are negative.

Diagnosis.—Multiple cartilaginous exostosis; stationary.

Case 2.—C. G., male, single; age 16 years; tool maker.

Complaint.—Lumps on leg and crooked leg.

Family History.—No evidence of heredity in parents or children.

Past History.—Scarlet fever at 4 years of age.

Present History.—Patient has noticed lumps upon various bones, all his life, some of which have grown during last 6 years. During last few years has noticed that the right knee was becoming badly knocked. Never has had pain.

Physical Examination.—A fairly tall, rather slimly built young man; narrow shoulders and hips and with a decided right knock-knee; fairly well developed muscularly and fairly well nourished; weight 126 lbs. The lungs, heart, and other organs are negative.

Examination of the bony skeleton shows numerous growths or nodules most noticeably above and inside of right knee, also left knee. Other growths are also found on forearms near lower end. These growths are not so easily felt or so hard as in Case 1. The growths above the knees, however, are easily grasped and even seen.

The radiographs show many irregular growths, some long and spindle-shaped, others flat, rounded, and more like lumps. It is evident that these are bony growths with considerable cartilage. They are not so dense or definite in outline as those seen in Case 1. They occur mainly near and over the epiphysis. The bones themselves are apparently normal with abnormal epiphyses. Growths are seen on nearly all the bones.

The urine and blood examinations are negative.

Diagnosis.—Multiple cartilaginous exostosis; progressive.

Calcium and Magnesium Balances.

The data relative to the calcium and magnesium balances in Cases 1 and 2 may be found in Tables I and II. Those for the normal individuals, Subjects 1 and 2, are given in the previous paper.[2] In the first period on a diet low in calcium and magnesium Case 1, the more advanced case of exostosis, behaved with respect to the calcium balance in a manner very similar to the normal subjects, being in slight negative balance. On the other hand, when the magnesium balance exhibited by this subject is examined it will be found different from that for the normal subjects. With the latter a positive balance was obtained in the first period, but with the abnormal subject there is a very significant negative equilibrium. In the growing youth, Case 2, the negative calcium balance in the first period is marked, being three times greater than in Case 1. On the other hand, the negative magnesium balance in this period is much smaller than in Case 1.

Addition of calcium in the form of milk in the second period resulted in an approach to calcium equilibrium in all four cases. However, the added calcium did not materially alter the magnesium balance in either case of exostosis.

[2] Underhill, Honeij, and Bogert,[1] Tables II and III.

TABLE I.

Case 1; Exostosis. Calcium and Magnesium Balances.

Period.	Diet.	Date.	Calcium. Intake.	Calcium. Output. Urine.	Calcium. Output. Feces.	Calcium. Balance.	Magnesium. Intake.	Magnesium. Output. Urine.	Magnesium. Output. Feces.	Magnesium. Balance.
		1917	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	Calcium-low and magnesium-low.	Jan. 23	573	180			366	110		
		" 24	537	418			362	125		
		" 25	563	175			354	114		
		" 26	500	321			336	136		
Total			2,173	1,094	1,938	−859	1,418	475	2,556	−1,623
				3,032				3,041		
Average per day			543	273	484	−214	354	118	639	−405
				758				760		
2	Calcium-high and magnesium-low.	Jan. 27	1,513	282			507	162		
		" 28	1,603	279			537	128		
		" 29	1,593	417			536	223		
Total			4,709	978	3,624	+107	1,580	513	2,507	−1,440
				4,602				3,020		
Average per day			1,569	326	1,208	+35	526	171	835	−480
				1,534				1,006		
3	Calcium-low and magnesium-low.	Jan. 30	483	188			345	92		
		" 31	450	138			322	151		
		Feb. 1	479	352			334	115		
Total			1,412	678	1,427	−693	1,001	358	2,401	−1,758
				2,105				2,759		
Average per day			470	226	475	−231	333	119	800	−586
				701				919		
4	Calcium-low and magnesium-high.	Feb. 2	517	386			550	177		
		" 4	522	129			573	118		
		" 4	522	217			553	180		
		" 5	516	279			554	207		
		" 6	459	161			541	141		
Total			2,536	1,172	1,418	−54	2,771	823	6,378	−4,430
				2,590				7,201		
Average per day			507	234	283	−11	554	164	1,275	−886
				518				1,440		

TABLE II.

Case 2; Exostosis. Calcium and Magnesium Balances.

Period.	Diet.	Date.	Calcium. Intake.	Calcium. Output. Urine.	Calcium. Output. Feces.	Calcium. Balance.	Magnesium. Intake.	Magnesium. Output. Urine.	Magnesium. Output. Feces.	Magnesium. Balance.
		1916	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	Calcium-low and magnesium-low.	July 17	229	219			142	123		
		" 18	213	370			131	144		
		" 19	167	463			122	118		
		" 20	224	404			133	140		
Total			833	1,456	2,299	−2,922	528	525	379	−376
				3,755				904		
Average per day			208	364	574	−730	132	131	94	−94
				938				226		
2	Calcium-high and magnesium-low.	July 21	920	436			166	128		
		" 22	897	331			146	116		
		" 23	918	448			156	91		
Total			2,735	1,215	1,752	−232	468	335	315	−182
				2,967				650		
Average per day			911	405	584	−77	156	111	105	−60
				989				216		
3	Calcium-low and magnesium-low.	July 24	213	339			151	82		
		" 25	210	615			143	126		
		" 26	235	609			141	120		
Total			658	1,563	2,211	−3,116	435	328	246	−139
				3,774				574		
Average per day			219	521	737	−1,038	145	109	82	−46
				1,258				191		
4	Calcium-low and magnesium-high.	July 27	210	799			353	160		
		" 28	182	259			342	86		
		" 29	228	615			349	181		
Total			620	1,673	1,557	−2,610	1,044	427	483	+134
				3,230				910		
Average per day			206	557	519	−870	348	142	161	+44
				1,076				303		

The return to a diet low in calcium and magnesium in Period 3 caused all four subjects to assume a negative calcium balance, the two normal individuals and Case 1 behaving very much alike. Case 2, on the other hand, shows a very large negative calcium balance. When the magnesium balance is considered in this period it is found that the two normal subjects and Case 2 behave similarly; that is, show a slight negative magnesium balance. Case 1, on the other hand, exhibits a marked negative magnesium balance.

Placed in the fourth period upon a relatively high magnesium diet the two normal subjects remain in a significant negative calcium balance. With Case 1 equilibrium is practically attained whether considered from the standpoint of a 3 or 5 day period. Case 2, however, exhibits a marked negative balance. Addition of magnesium in the fourth period causes both normal subjects and Case 2 to change from a negative magnesium balance to a positive balance. In contrast to these results Case 1 exhibits an even greater negative balance than on a magnesium-free diet. This is true whether considered from the view-point of a 3 or 5 day period.

These facts demonstrate that in the exostosis cases there is a considerable variation in the manner in which the two subjects dispose of calcium and magnesium. Case 1 must be regarded clinically as a stabilized case of exostosis. This fact was demonstrated by comparative examinations 1 year apart. Case 2 is considered as in the progressive stage of the disease. From the metabolic view-point Case 1 behaves very much like a normal subject with respect to calcium exchange. A comparison of magnesium metabolism in this subject with that in the normal individuals shows that a significant negative balance is always present whether the subject is kept upon a low or high calcium or magnesium diet.

With the growing youth, Case 2, the calcium balance is always markedly negative whether the calcium intake is high or low. In other words, it would appear that this organism constantly attempted to rid itself of calcium. Negative magnesium balances are the rule also with this individual, except in the period of high magnesium intake. On the other hand, the negative magnesium is not at all comparable with that obtained with the stabilized case, No. 1.

These facts lead to the hypothesis that in exostosis in the progressive stage the organism shows an active effort to resist storage of calcium and magnesium. With the latter element, however, this tendency is not so marked. In the stabilized stage calcium exchange appears to approach the normal condition, but the loss of magnesium from the body is quite notable.

Further Analysis of the Data.

Calcium Metabolism.

The data obtained from the study of exostosis have been subjected to a further analysis in order to determine the correctness of the hypothesis stated above; namely, that in general in exostosis calcium and magnesium are eliminated from the body rather than stored.

Excretion of Added Calcium.—Calculation of the percentage of added calcium (Table III) eliminated from the organism in stabilized

TABLE III.

Excretion of Added Calcium.

Subject.	Excretion of added calcium.*
	per cent
Subject 1; normal	100+
" 2; "	92+
Case 1; exostosis	96+
" 2; "	?

* Period 2; calcium added as milk.

exostosis demonstrates a great similarity to that excreted by normal individuals under like experimental conditions. The percentage excretion of calcium in the growing patient, Case 2, is impossible to compute owing to the fact that the increased calcium intake appeared to check temporarily the excessive excretion of calcium characteristic of the subject upon a low calcium intake. In Periods 3 and 4 the massive excretion again prevails.

Relation of Calcium Output to Intake.—In Table IV may be found ratios of calcium excreted to calcium ingested for the four experimental

periods. These figures demonstrate that the stabilized case of exostosis behaved in a manner very similar to normal individuals. In Case 2, a developing boy without marked deposition of bone in growths, the metabolic rate is evidently different, since in the initial calcium-poor period the calcium output was more than four times the intake. It is, of course, possible that the low calcium intake acted as a stimulus to elimination of calcium from the body. Be this as it may, it is certain that when calcium was added to the diet in Period 2 the ratio is the same as that of normal individuals. In other words, this subject apparently had not lost the ability of disposing of calcium in a normal manner. When the addition of calcium

TABLE IV.

Relation of Calcium Output to Calcium Intake.

Ratio: $\frac{\textit{Calcium Output}}{\textit{Calcium Intake}}$.

Subject.	Period.			
	1	2	3	4
Subject 1; normal	1.6	1.0	1.8	1.9
" 2; "	1.2	0.9	1.7	1.7
Case 1; exostosis	1.4	0.9	1.4	1.0
" 2; "	4.2	0.9	5.7	5.2

to the diet was discontinued the old ratio of output to intake again appeared, but to an even more exaggerated degree. It is probable that this is an expression of delay or lag in excretion, calcium either being retained somewhat temporarily or its introduction checking for a time the tendency to excessive elimination.

From the data it is evident that under the experimental conditions in active exostosis the organism is not tenacious of calcium. This fact may indicate that in its initial stages progress of the disorder may possibly be checked by proper restriction of the intake of calcium. It is, of course, impossible to draw such a deduction from a single instance, but the behavior of this individual under the experimental conditions imposed at least warrants the pursuit of such a lead.

Relation of Output to Intake for the Entire Period.—The relation of the total intake of calcium to its total output over the entire experimental period emphasizes the points discussed above (Table V).

Case 1, the stabilized case of exostosis, behaved very much like a normal individual. On the other hand, Case 2, the growing patient, showed a massive excretion.

TABLE V.

Relation of Calcium Output to Intake for the Entire Period.

Subject.	Calcium		
	Output.	Intake.	Difference.
	mg.	*mg.*	*mg.*
Subject 1; normal	10,443	7,583	−2,860
" 2; "	12,626 (13 days).	9,752	−2,874
Case 1; exostosis	11,293 (13 ").	9,815	−1,478
" 2; "	13,726	4,846	−9,880

Relation of Calcium Intake and Output to Body Weight.—Inspection of Table VI will demonstrate at once the same general features of calcium metabolism already discussed. On the basis of weight Case 2 eliminated more calcium than any other individual, and yet the intake of calcium was not much more than one-half that of the others. Variations in body weight therefore will not account for the massive excretion observed with Case 2.

TABLE VI.

Relation of Calcium Intake and Output to Body Weight.

Subject.	Body weight.	Calcium.		Calcium per pound of body weight.	
		Intake.	Output.	Intake.	Output.
	lbs.	*mg.*	*mg.*	*mg.*	*mg.*
Subject 1; normal	105	7,583	10,443	72	99
" 2; " (13 days)	150	10,290	13,316	68	88
Case 1; exostosis	112	9,815	11,293	87	100
" 2; "	132	4,846	13,726	36	104

Absorption of Calcium.—Under the limitations outlined in the previous paper absorbed calcium is defined as the calcium eliminated through the urine; unabsorbed calcium that passing out with the feces. It may be seen from Table VII that the well advanced case of exostosis, Case 1, behaves like a normal person. On the other hand, with the growing boy, Case 2, the output of calcium through the urine on the calcium-poor diet (Period 1) was more than 1.5 times the intake, showing a distinct loss of calcium from the body tissues. Nevertheless, on a calcium-rich diet (Period 2) this individual exhibits a behavior similar to that of a normal person, but when he was again placed upon a calcium-poor diet (Periods 3 and 4) the calcium

TABLE VII.

Relation of Urinary Calcium Excretion to Calcium Intake.

$$\textit{Ratio: } \frac{\textit{Calcium of Urine}}{\textit{Calcium of Food}}.$$

Subject.	Period.			
	1	2	3	4
Subject 1; normal	1.0	0.4	1.0	1.0
" 2; "	0.4	0.2	0.5	0.8
Case 1; exostosis	0.5	0.2	0.4	0.4
" 2; "	1.7	0.4	2.3	2.7

excreted by the urine was more than twice that ingested. This excessive excretion occurred in spite of the fact that there was in this individual an enormous quantity of cartilage being laid down and that there would be accordingly a great demand for bone salts.

Relation of Fecal Calcium to Calcium Intake.—A consideration of the relation of calcium in the feces to calcium ingested shows that in the exostosis patients the same type of behavior as discussed above holds true (Table VIII). The only deviation from the normal excretion of calcium by way of the feces to be observed is that in Case 2. On a calcium-low diet (Period 1) more than 2.5 times the calcium of the food is eliminated by way of the intestine. This does not indicate any inability to absorb calcium, for on a calcium-rich diet (Period 2) the calcium excreted through the feces assumes a

relation to the calcium of the food of normal proportions. Resumption of a calcium-poor diet causes the reappearance of the same unusual degree of calcium excretion observed in the first period.

TABLE VIII.

Relation of Fecal Calcium to Calcium Intake.

$$\textit{Ratio: } \frac{\textit{Calcium of Feces}}{\textit{Calcium of Food}}.$$

Subject.	Period.			
	1	2	3	4
Subject 1; normal	0.6	0.6	0.8	0.8
" 2; "	0.8	0.7	1.1	0.8
Case 1; exostosis	0.9	0.7	1.1	0.5
" 2; "	2.7	0.6	3.3	2.5

Relation of Urinary Calcium to Fecal Calcium.—If a comparison is made between the output of calcium in the urine and feces it is apparent (Table IX) that in the cases of exostosis this relation does not deviate very markedly from that of normal individuals.

TABLE IX.

Relation of Urinary Calcium to Fecal Calcium.

$$\textit{Ratio: } \frac{\textit{Calcium of Urine}}{\textit{Calcium of Feces}}.$$

Subject.	Period.			
	1	2	3	4
Subject 1; normal	1.5	0.6	1.1	1.1
" 2; "	0.5	0.2	0.4	1.0
Case 1; exostosis	0.5	0.2	0.4	0.8
" 2; "	0.6	0.6	0.7	1.0

In a stabilized case of multiple exostosis the calcium exchange differs little from that of a normal individual whether the abnormal subject is maintained upon a calcium-poor diet or upon one rich in this element. In a growing individual with multiple exostosis the calcium exchange is markedly different from that of a normal indi-

vidual. On a calcium-poor diet calcium is lost from the body in relatively large quantity, being eliminated by both the urine and feces in a perfectly normal ratio. On a calcium-rich diet calcium is retained to an extent not widely deviating from that in normal persons. A resumption of a calcium-poor diet again induces excessive calcium elimination. These facts suggest a possible dietary procedure to check the further development of the abnormal condition through deposit of calcium in early cartilaginous growths.

Magnesium Metabolism.

Excretion of Added Magnesium.—The figures in Table X show how promptly added magnesium is excreted in subjects with multiple exostosis. Under like experimental conditions normal individuals

TABLE X.

Excretion of Added Magnesium.

Subject.	Excretion of added magnesium.*
	per cent
Subject 1; normal	36
" 2; "	45
Case 1; exostosis	100
" 2; "	80

* Period 4; magnesium added as citrate.

retain more than one-half the added magnesium, whereas in the abnormal cases the excretion is much larger. With the stabilized case of exostosis, Case 1, 100 per cent of the added magnesium was quickly eliminated. The less advanced case, Case 2, excreted about 80 per cent of the introduced magnesium. This may perhaps be explained by the view that the stabilized case had no unusual demand for bone-building salts, whereas in the young, growing, active subject, the calcification of growths had not progressed to a marked degree.

Relation of Magnesium Output to Intake.—Considerable interest attaches to the relation which exists between magnesium output and intake, especially in Case 1, the well stabilized case of exostosis

(Table XI). On a diet low in magnesium the output is twice as great as the intake and indeed at times, as in Period 3, may be almost three times as great. Added magnesium does not change this relation. This type of case, therefore, either is unable to retain magnesium or else the body attempts to rid itself of it. The latter view is undoubtedly the more probable explanation, since the magnesium excretion is constantly three times greater than the intake.

With the less advanced case, Case 2, the same type of behavior relative to magnesium may be observed, although the degree of excretion is not so marked. This case in general more nearly approaches normal behavior, and when magnesium is added there is

TABLE XI.

Relation of Magnesium Output to Intake.

Ratio: $\frac{\textit{Magnesium Output}}{\textit{Magnesium Intake}}$.

Subject.	Period.			
	1	2	3	4
Subject 1; normal	0.9	0.9	1.2	0.7
" 2; "	0.9	1.1	1.0	0.8
Case 1; exostosis	2.1	1.9	2.7	2.6
" 2; "	1.6	1.4	1.3	0.9

distinct retention. This fact leads to the query whether the results obtained with this subject are explicable on the hypothesis that the calcium intake was too small for the individual to be able to reach equilibrium. Such a view is pertinent, since clinically the patient was in apparent need of both magnesium and calcium for the maintenance of general growth. Moreover, the new growths were abundant and progressive. The fact that added calcium brought this subject into balance favors the hypothesis stated above. On the other hand, distinctly against this view-point is the fact that as the experimental period advanced, equilibrium was more nearly approached, although the magnesium intake became progressively lower. Inasmuch as inadequate magnesium intake cannot be ascribed as the cause of the peculiar behavior of Case 1, the stabilized case, and, moreover, since the general features of both cases are very simi-

lar, it seems logical to conclude that the deviation from normal observed in the less advanced case, Case 2, is to be attributed to the stage of the disease rather than to an inadequate magnesium supply.

Relation of Output to Intake for the Entire Period.—The figures in Table XII, showing the relation of magnesium output to intake for the total experimental period, merely corroborate and emphasize the points discussed above.

TABLE XII.

Relation of Magnesium Output to Intake for the Entire Period.

Subject.	Magnesium.		
	Output.	Intake.	Difference.
	mg.	*mg.*	*mg.*
Subject 1; normal	4,133	4,468	+335
" 2; " (13 days)	5,806	5,894	+88
Case 1; exostosis (13 ")	13,140	5,661	−7,479
" 2; "	3,038	2,475	−563

Relation of Magnesium Intake and Output to Body Weight.—An inspection of Table XIII will show at once that the variability of body weight will not explain the differences observed previously with respect to magnesium exchange in multiple exostosis. Moreover, when the intake and output of magnesium are considered in relation to body weight it is at once apparent that insufficient intake cannot explain the variations. Thus, Case 1 ingested a relatively greater quantity of magnesium than any of the other subjects and yet the

TABLE XIII.

Relation of Magnesium Intake and Output to Body Weight.

Subject.	Body weight.	Magnesium.		Magnesium per pound of body weight.	
		Intake.	Output.	Intake.	Output.
	lbs.	*mg.*	*mg.*	*mg.*	*mg.*
Subject 1; normal	105	4,468	4,133	42	39
" 2; " (13 days)	150	5,894	5,806	39	38
Case 1; exostosis (13 ")	112	5,661	13,140	50	117
" 2; "	132	2,475	3,038	18	23

excretion was enormous. It is perhaps possible that with Case 2 a small intake of magnesium may play a part in the results noted. On the other hand, this seems hardly probable, since in general the behavior of this individual with respect to magnesium exchange resembled that of Case 1.

Absorption of Magnesium.—A comparison of the relation existing between urinary magnesium and food magnesium in normal individuals and in cases of multiple exostosis shows a striking similarity in all instances except one; namely, Case 2. Here the urinary magnesium excretion on diets low in magnesium varies from 0.7 to 0.9 of the intake instead of 0.3 to 0.4 as with normal subjects (Table XIV). Addition of magnesium to the diet causes this relation to

TABLE XIV.

Relation of Urinary Magnesium Excretion to Magnesium Intake.

$$\textit{Ratio: } \frac{\textit{Magnesium of Urine}}{\textit{Magnesium of Food}}.$$

Subject.	Period.			
	1	2	3	4
Subject 1; normal	0.3	0.3	0.4	0.2
" 2; "	0.3	0.3	0.3	0.2
Case 1; exostosis	0.3	0.3	0.3	0.3
" 2; "	0.9	0.7	0.7	0.4

approach the higher limits exhibited by the normal subjects. From these facts it is evident that in the abnormal cases under observation magnesium absorption was not inferior to that in normal individuals.

Relation of Fecal Magnesium to Magnesium Intake.—A consideration of the relation of excretion of magnesium by way of the feces to ingestion of this element shows that only in Case 1 is the ratio of fecal magnesium to food magnesium outside the normal limits (Table XV). With Case 1 there is a massive excretion of magnesium by way of the intestine. Inasmuch as this excretion is far greater than the intake, and since the absorption of magnesium is normal, it follows that the excess magnesium eliminated comes from the tissues, the intestine being the principal path of elimination.

TABLE XV.

Relation of Fecal Magnesium to Magnesium Intake.

$$\textit{Ratio: } \frac{\textit{Magnesium of Feces}}{\textit{Magnesium of Food}}.$$

Subject.	Period.			
	1	2	3	4
Subject 1; normal	0.5	0.6	0.8	0.4
" 2; "	0.6	0.8	0.7	0.5
Case 1; exostosis	1.8	1.5	2.9	2.3
" 2; "	0.7	0.7	0.6	0.5

Relation of Urinary Magnesium to Fecal Magnesium.—From the data in Table XVI it is apparent that the ratio of urinary magnesium to magnesium of the feces is different from that exhibited by normal persons. In the normal subjects approximately twice as much magnesium is excreted by way of the intestine as through the urine. This relation holds fairly constant whether the individual is maintained upon a low magnesium diet or whether magnesium has been added to the diet. In the stabilized case of exostosis, Case 1, the fecal excretion of magnesium is five to seven times that of the urinary elimination. With the less advanced case, Case 2, conditions are reversed. The urinary excretion of magnesium exceeds somewhat the elimination by way of the feces with a single exception. This occurs in Period 4, during which magnesium citrate was added to the

TABLE XVI.

Relation of Urinary Magnesium to Fecal Magnesium.

$$\textit{Ratio: } \frac{\textit{Magnesium of Urine}}{\textit{Magnesium of Feces}}.$$

Subject.	Period.			
	1	2	3	4
Subject 1; normal	0.6	0.5	0.5	0.6
" 2; "	0.5	0.4	0.4	0.4
Case 1; exostosis	0.2	0.2	0.1	0.1
" 2; "	1.4	1.1	1.3	0.8

food. Under these circumstances magnesium elimination through the feces was slightly higher than excretion through the urine. In this period, therefore, Case 2 approaches quite closely the normal type in excretion of magnesium. From the observations it is evident that in multiple exostosis there is a derangement of the excretory ratio for magnesium, the deviation from normal being in either direction. Thus, in stabilized multiple exostosis magnesium is lost from the body principally by the intestine. When the progress of the disease has not become stabilized it is indicated that the greater portion of magnesium excreted goes by way of the kidneys.

In a stabilized case of multiple exostosis magnesium added to a diet low in this element is promptly excreted. Magnesium elimination in a less advanced case follows a similar course but to a less marked degree. These facts may be interpreted to mean that in the stabilized case there was no unusual demand for bone-building salts, whereas in the more active stage of the disease the degree of calcification in growths had not progressed to a marked extent, hence more magnesium was retained. In the stabilized case of exostosis, magnesium output was two to three times greater than the intake whether the subject was maintained upon a diet poor or rich in magnesium. With the less advanced case the ratio of excretion to intake follows closely that of the stabilized case, but the degree of excretion is not so marked. When magnesium is added there is distinct evidence of retention. The variations in magnesium exchange cannot be attributed to variable body weight. The absorption of magnesium in individuals with multiple exostosis is not inferior to that in normal subjects. Inasmuch as neither inadequate intake nor faulty absorption will account for the excessive elimination of magnesium in multiple exostosis it is obvious that it has its origin in the body tissues. From the observations detailed above it is apparent that in multiple exostosis there is a derangement of the excretory ratio for magnesium, the deviation from normal being in either direction. In the stabilized condition magnesium is lost from the body principally by way of the intestine, whereas in the more active stage of the disease the larger portion of magnesium excreted is by the kidneys.

Relation of Calcium to Magnesium.

Relation of Calcium and Magnesium in the Food.—In a consideration of the metabolism of either calcium or magnesium it is of importance to determine the relation existing between these two elements in the food and in the excretion, since there is some evidence that the intake of one may influence the elimination of the other. It is at once apparent from Table XVII that in the first period all four subjects exhibited a strikingly constant ratio of calcium to magnesium in the food. When calcium was added to the diet, only in Case 2 was there a deviation. Apparently in this instance the calcium intake was greatly increased without a corresponding augmentation of magnesium. Throughout the remainder of the investigation the ratio is not notably peculiar in any instance. It is therefore evident that, in general, all subjects received approximately the same proportions of calcium and magnesium.

TABLE XVII.

Relation of Calcium and Magnesium Intake.

Ratio: $\dfrac{\textit{Calcium of Food}}{\textit{Magnesium of Food}}$.

Subject.	Period.			
	1	2	3	4
Subject 1; normal	1.3	3.6	1.2	0.8
" 2; "	1.4	3.1	1.2	0.9
Case 1; exostosis	1.3	3.0	1.4	0.9
" 2; "	1.5	5.8	1.5	0.6

Relation of Calcium and Magnesium in the Urine.—From an inspection of Table XVIII it may be seen that although the calcium-magnesium ratio of the food of all subjects was fairly constant a marked variation occurs even in the normal subjects in the relation of calcium and magnesium in the urine. With the stabilized case of exostosis the variations observed are within normal limits. Case 2, however, shows constantly a decided tendency to go beyond the normal limits. In general, the ratio of calcium to magnesium is higher than with the other subjects.

TABLE XVIII.

Relation of Calcium and Magnesium in the Urine.

$$\text{Ratio: } \frac{\text{Calcium of Urine}}{\text{Magnesium of Urine}}.$$

Subject.	Period. 1	2	3	4
Subject 1; normal	3.9	4.5	2.9	2.7
" 2; "	1.8	1.9	2.1	3.1
Case 1; exostosis	2.2	1.9	1.9	1.4
" 2; "	2.7	3.6	4.7	3.9

Relation of Calcium and Magnesium in the Feces.—The stabilized case of exostosis, Case 1, shows that magnesium is eliminated by the intestine in excessive amounts when considered relative to the calcium excretion (Table XIX, Period 1). Addition of calcium to the diet changes the ratio so that it is more nearly comparable with the normal ratio, although still far from it. This change is effected by the additional calcium eliminated and without any marked influence

TABLE XIX.

Relation of Calcium and Magnesium in the Feces.

$$\text{Ratio: } \frac{\text{Calcium of Feces}}{\text{Magnesium of Feces}}.$$

Subject.	Period. 1	2	3	4
Subject 1; normal	1.5	3.7	1.2	1.4
" 2; "	1.8	3.0	2.1	1.3
Case 1; exostosis	0.8	1.4	0.6	0.2
" 2; "	6.0	5.5	9.0	3.2

upon the magnesium excretion. Resumption to a low calcium diet again causes a return to the previous ratio. Addition of magnesium (Period 4) does not influence the output of calcium in the feces, and hence the ratio of calcium to magnesium is significantly decreased.

When the ratio of calcium to magnesium in the feces is considered with Case 2 the results are just the opposite of those observed with

Case 1. In other words, calcium is excreted by the intestine in excessive amounts (Period 1), the ratio not being materially altered even on a calcium-rich diet (Period 2). On returning to a calcium-poor diet (Period 3) again the ratio is greatly increased, apparently an indication of a lag in the rate of excretion. Addition of magnesium to the diet (Period 4) considerably lowers the ratio. This is caused by two factors, a somewhat lower calcium excretion and an increased magnesium output by the intestine.

The very diverse behavior exhibited by these two cases of exostosis leads one to query whether this difference is indicative of two stages in the abnormal metabolic process. That is, in the early stage calcium is more readily eliminated from the body than magnesium. In the stabilized condition, on the other hand, the reverse is true. Calcium is retained in a more or less normal manner but the relative excretion of magnesium is excessive. It is possible that in these two stages calcium and magnesium play a distinctive part in the calcification of the new growths, one element being more in demand at one period than at another.

The observations recorded above indicate that in general the relation of calcium and magnesium in the dietary of the experimental subjects was approximately the same for all subjects. Variations in excretion, therefore, cannot be explained on the hypothesis that varying proportions of one or the other element are responsible for the alterations in elimination previously noted. In general, in the early stage of exostosis calcium is excreted much more freely than magnesium, whereas in the stabilized stage of the disease magnesium is eliminated in excessive amount. In the stabilized case of exostosis the excessive excretion of magnesium is manifested through the intestinal path. On the other hand, with the less advanced case, Case 2, excessive elimination of magnesium is seen in both the urine and the feces. Neither in normal individuals nor in the cases of exostosis studied does addition of calcium or magnesium materially influence the excretion of the other element.

CONCLUSIONS.

In the stabilized stage of exostosis calcium exchange differs little from that of a normal individual whether the abnormal subject is maintained upon a calcium-poor diet or upon one rich in this element.

In the progressive stage of the disease calcium metabolism is markedly different from the normal in that calcium is lost from the body in large amounts when the subject is maintained upon a calcium-poor diet. This excessive elimination of calcium is by way of both the urine and feces in a normal ratio. When placed upon a calcium-rich diet calcium is retained to an extent not widely deviating from that obtained in normal subjects. A resumption to a calcium-poor diet again induces excessive calcium elimination.

In the stabilized stage of exostosis magnesium excretion is two or three times greater than the intake whether the subject is maintained upon a diet poor or rich in magnesium.

In the progressive stage of the disease the general type of magnesium excretion resembles that of the stabilized stage but the degree of elimination is not so marked.

Magnesium added to the diet in the stabilized stage is promptly excreted. The same test applied to the progressive stage gives evidence of retention of some magnesium. The degree of retention, however, is much less than that shown by normal individuals.

Absorption of both calcium and magnesium in exostosis is not inferior to that of normal subjects.

The facts enumerated suggest that in the early stages of exostosis, that is during the proliferative cartilage changes, the progress of the disease may perhaps be checked by proper dietary procedure—restriction of calcium and magnesium intake.

A FURTHER EXPERIMENTAL STUDY ON EXCITATION OF INFECTIONS OF THE THROAT.

By SAMUEL B. GRANT, STUART MUDD, and ALFRED GOLDMAN.

(*From the Department of Pathology of Washington University Medical School, St. Louis.*)

(Received for publication, April 1, 1920.)

In experiments previously published it has been shown that chilling of the body surface causes vasoconstriction and ischemia—not, as theretofore assumed, congestion—in the mucous membranes of the palate, pharynx, and palatine tonsils.[1] Upon rewarming the subject this ischemia was shown to disappear only partially from the palate and pharynx, but, in a single experiment upon the palatine tonsil, promptly gave way to an actual hyperemia. Because of the peculiar importance of the tonsils as foci of infection, it was considered necessary to devote further study to their reactions. The present experiments amply confirm the occurrence in the tonsils of the reaction of ischemia to chilling of the body surface; a composite of the four recovery curves obtained bears out also the first observation that the tonsils, upon the subject's being rewarmed after chilling, show a considerably greater tendency than do the contiguous mucous membranes of the palate and pharynx to regain a full blood supply.

Concurrent bacteriological observations on the pharynx and tonsils of several of the subjects showed after the experiments in several instances apparently an increase in the relative number of some of the pathogenic bacteria, synchronous with the appearance of a clinical sore throat.

Urine analysis indicated secretion during an experiment of a more dilute and less acid urine. In no instance was albuminuria or glycosuria produced.

[1] Mudd, S., and Grant, S. B., *J. Med. Research*, 1919, xl, 53.

Method.

The thermopile method described in the earlier paper[2] was again used, but with several notable improvements in detail. The subject entered the experimental room, kept between 16.9° and 20.9°C., warmly wrapped in loose garments. Thoracic and abdominal pneumographs were adjusted to a moving drum writing in the subject's view. The terminals of a thermopile, borne on a carrier of galvanized iron wire, were strapped by adhesive tape to the subject's forehead. The terminals of a second thermopile were fixed by means of another galvanized iron applicator and a specially devised holder, to be described below, in apposition with the mucous membrane of one palatine tonsil. Thereafter the subject maintained his position as nearly as possible constant throughout the experiment. The proximal ends of the thermopiles were kept packed in cotton in a test-tube with the bulb of a sensitive thermometer, and were connected through a rocking key with a d'Arsonval galvanometer.

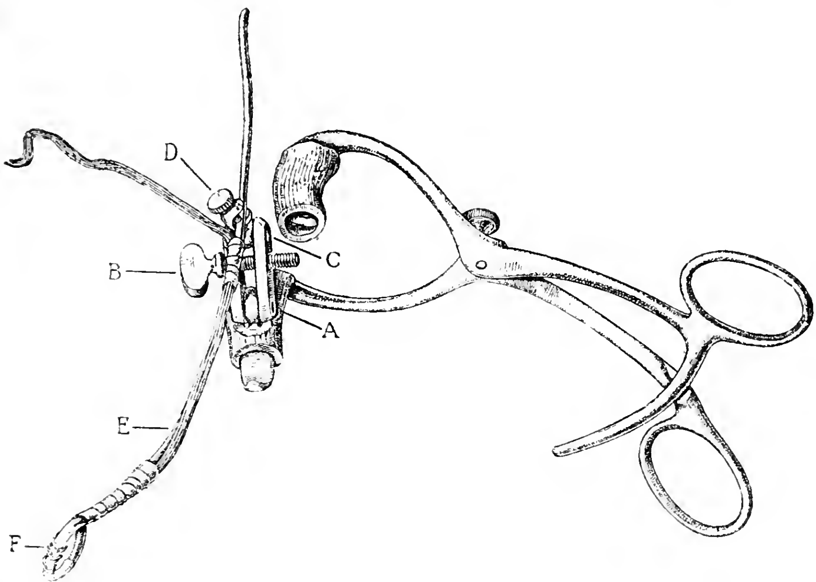
The subject's nostrils were plugged with cotton; breathing was through the open mouth. At least a part of the difficulty introduced by the excessive secretion of saliva under these circumstances was eliminated in the present experiments by keeping in the subject's mouth a glass saliva ejector of the type used by dentists, connected through a rubber tube to a suction pump on a water faucet.

When thermopiles, pneumographs, and saliva tube had once been properly adjusted, readings of galvanometer and thermometer were made at about 60 second intervals for a few minutes. Respiration was then deepened by an amount sufficient to increase the excursion of the pneumograph levers on the average for the experiments illustrated in the charts by about 73 per cent, the respiratory rate being kept constant throughout the experiment with the aid of a metronome. With the intake of cool room air thus increased, mucous membrane temperature usually fell slightly. When the readings showed themselves approximately constant, the subject's wraps were all removed and an electric fan was turned at short range on the lumbar region of the back; chilling continued in the experiments illustrated in the charts from 7 to 19 minutes, with an average duration

[2] Mudd and Grant,[1] pp. 54–60.

of 13.8 minutes. The subject was finally rewrapped and readings were continued until the end of the experiment.

From the thermometer and galvanometer readings thus made, skin and mucous membrane surface temperatures are readily computed and their changes accurately followed. It is to be borne in mind that these temperature changes under the conditions of the experiments have been shown to be satisfactory indices of local vasomotor changes.[3] Therefore, although the curves in the text-figures actually record



TEXT-FIG. 1. Applicator holder with tonsillar applicator and thermopile in position. *A* and *C*, ball and socket joints made by metal spheroids and blades of head mirror; *B*, set screw for tightening joints at *A* and *C*; *D*, set screw for fastening applicator in holder; *E*, galvanized iron wire applicator; *F*, applying surface bearing thermopile terminals.

variations in superficial temperature of the skin and exposed mucous membranes, what they indicate is alteration in vasomotor tone—a fall in temperature means local vasoconstriction and ischemia, a rise local vasodilation.

The most important improvement in method in the present study has been the devising of a special instrument by which accurate and

[3] Mudd and Grant,[1] pp. 58–60.

stable adjustment of the thermopile terminals to the tonsillar surface, or other site in mouth or throat, can be effected. Into one arm of a Doyen mouth gag (Text-fig. 1) a small brass spheroid was screwed. A second brass spheroid bearing a groove closed by a set screw (*D*) was made. The two spheroids were connected by means of the blades of an ordinary head mirror (*C*). The blades could be clamped stably upon the spheroids by means of a second set screw (*B*). The applicator (*E*) so shaped as to have its tip fit against the tonsil, was held in place in the groove by the set screw *D*. By varying the shape and position of the applicator and arranging properly the joints at *A* and *C* any desired application could be made. The subject's teeth were protected from the metal gag by rubber, as shown in the drawing.

As is evident, the iron wire applicators used with this new holder are somewhat simpler than the original types; they are also of slightly lighter wire; No. 13 has been found most satisfactory.

The subjects used, as in the earlier experiments, were all upper class medical students, except J. D. R. and B. J. F. in the first and W. G. E. in the present series, who were laboratory employees several years younger.

For calibration the unknown temperature terminals of the thermopiles were bound with elastic about the bulb of a sensitive thermometer, which was immersed in a suspended test-tube of distilled water or salt solution. In the earlier experiments heat in small amounts was applied at short intervals directly to the test-tube. The contained liquid was stirred, and, when equilibrium had been reached, the galvanometer deflection and temperatures of the two ends of the thermopiles were read. From these data the calibration curves for the thermopiles were constructed. Since these were apparently, within the temperature range of the experiments, virtually straight lines, the results were averaged for each thermopile and the calibration constants thus obtained; *i.e.*, the straight line $x = ky$, when y is the galvanometer deflection in millimeters, x the temperature difference in °C. between the two ends of the thermopiles, and k the calibration constant, in each case with a value of approximately 0.1.

In the experiments of the present series the test-tube of water containing the thermopile terminals and the thermometer was immersed in addition in a suspended beaker of water containing a stirrer, and heat was applied to the beaker at intervals. The water in the beaker and that in the test-tube were then stirred until a constant temperature had been reached, and the thermometer and galvanometer readings then taken. With the more accurate results thus obtained,

the calibration curves were found to deviate appreciably, though very slightly, from straight lines. Temperatures were therefore taken from the curves directly. The deviations were such that with the larger deflections the temperatures calculated from the calibration constant were slightly higher than those actually shown by the curve; with the smaller deflections calculated temperatures were slightly too low. For instance, to take extreme values, with a galvanometer deflection of 206 mm., the thermopile constant temperature end being 17.75°C., the mucous membrane temperature calculated from the calibration "constant" (in this case 0.09904) is 38.15°C., that shown by the curve, 37.43°C. With a galvanometer deflection of 56 mm. and constant end at 18.5°, the calculated mucous membrane temperature is 24.05°, that shown on the curve is 24.47°C. The high mucous membrane temperature values mentioned previously[4] are thus at least in large part accounted for. To be corrected the curves of the first paper should be very slightly foreshortened along the vertical axis; however, the error introduced was obviously quite negligible from the point of view for which the curves were designed; *i.e.*, to show temperature variations through a small range.

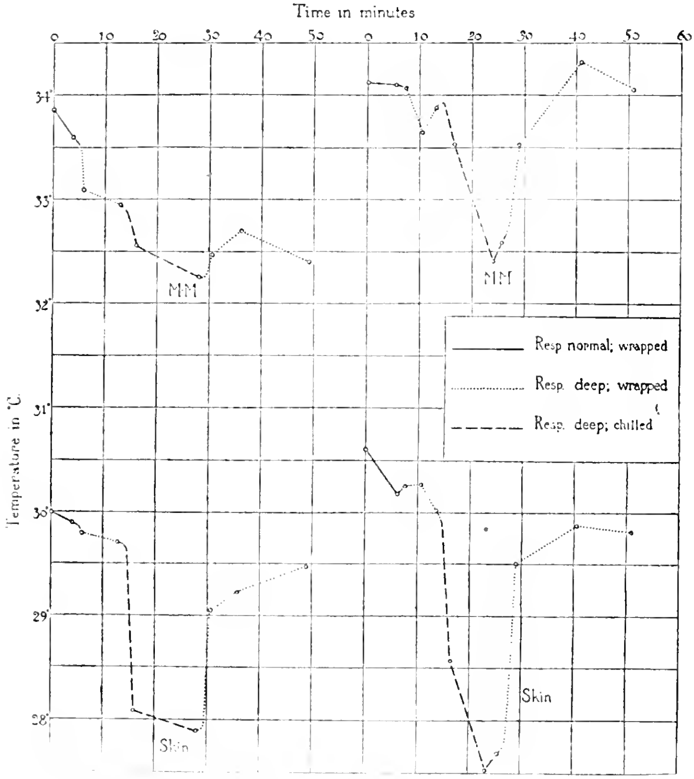
Vasomotor Reactions of Palatine Tonsils.

Composite Graphs.—The characteristic reactions of skin and mucous membrane to chilling of the body surface are shown in Text-fig. 2. The curves on the right are a composite of four experiments upon three different subjects; for the mucous membrane curve the thermopile tips were applied to the palatine tonsil surface; the synchronous lower curve is from the skin of the forehead. The curves on the left are a composite of three experiments upon the forehead, and, in two instances, mucous membrane of soft palate, in one instance, mucous membrane of oropharynx; two subjects were used. In each case the first and last point of the experiment, the point immediately before and after a change of conditions, and the point of maximum mucous membrane response to rewrapping were taken, and from their average values the curves were drawn. In the right hand curve the two points of maximum mucous membrane response to deepened respiration and to chilling are also included.

The palatine-oropharyngeal curve underwent the usual slight depression with deepened respiration. Upon chilling, the mucous membrane temperature fell 0.69°C. in 14 minutes; the corresponding point on the skin curve shows a depression of 1.80°. Upon rewrapping,

[4] Mudd and Grant,[1] p. 55.

the mucous membrane temperature rose in 7.3 minutes 0.40°, thereafter sinking slightly. The skin curve showed a maximum rise of 1.60° in 14.8 minutes. The maximum recovery exhibited within a 20 minute period after rewrapping was, then, for the palatine-oropharyngeal curve only 58 per cent of the fall in response to chilling; for the skin curve recovery was 88.8 per cent of the fall.



TEXT-FIG. 2. Reactions to chilling and to rewarming. On left, temperatures of skin and mucous membranes (*M.M*) of soft palate and oropharynx (composite graphs of Experiments 10, 12, and 13); on right, temperatures of skin and palatine tonsil (composite graphs of Experiments 1, 3, and 5 of the present series and Experiment 20 of the earlier series[5]).

---

[5] Mudd and Grant,[1] p. 85.

The palatine mucous membrane and skin curves responded to chilling by the usual depression, amounting to 1.47° for tonsil and 2.49° for skin. On rewarming, the mucous membrane reached a maximum rise of 1.72° in 14.1 minutes, the skin a rise of 2.33° in 14.5 minutes. Recovery was thus 117 per cent for tonsillar mucous membrane, 93.6 per cent for skin.

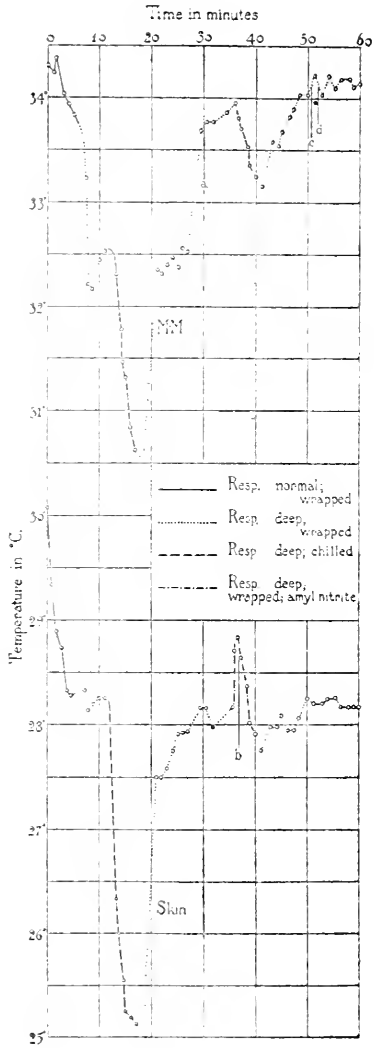
It is to be noted that, because the subject could not change position during the experiment and because of all the apparatus attached to him, he was practically never quite so warmly wrapped after chilling as he had been before. If conditions had been made identical before and after, doubtless all the recovery curves would have been somewhat higher.

Evidently the tonsils tend strongly to react to rewarming after chilling by becoming hyperemic, whereas the pharynx and palate, as was also shown in the earlier experiments, tend to remain somewhat ischemic for a considerable period after chilling. The skin occupies an intermediate position and returns approximately to its original condition.

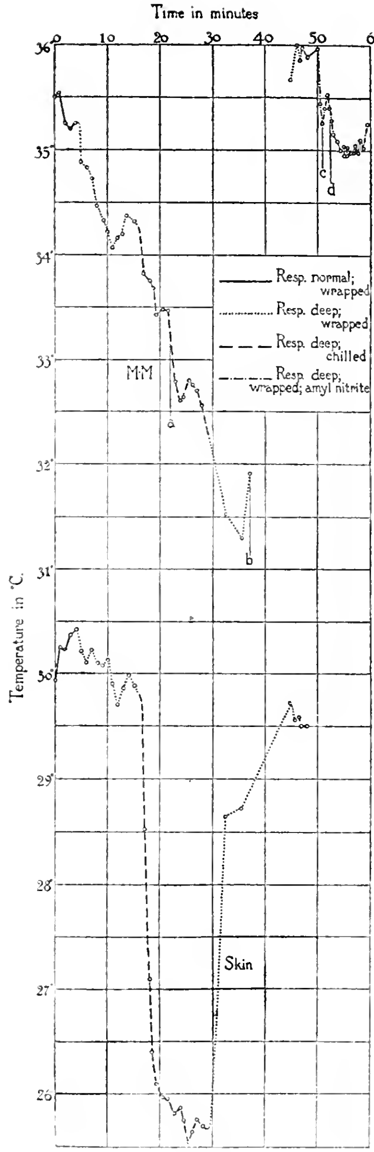
Experiment 1.—Subject 1, W. G. E. June 11, 1919, 11 a.m. to 12 m. Mucous membrane thermopile on left tonsil. Skin thermopile on forehead. Respirations 14 per minute. Mouth open; nostrils plugged. Thoracic and abdominal pneumographs. Room temperature 17.30–18.05°C.

Text-fig. 3 shows strikingly the characteristic tonsillar reactions. When the mucous membrane temperature had adjusted itself to deep respiration, chilling was begun at 0:12.[6] By 0:17 the mucous membrane temperature had fallen 1.91°, the skin temperature 3.11°. The subject was rewrapped at 0:17.5. 17 minutes later the tonsillar temperature had risen 3.24°, the skin temperature 3.04°. At 0:35 inhalation of an ampule of amyl nitrite was begun. The short transient rise in skin temperature, corresponding to the visible flush, at once developed; the peak, 0.66° higher, was reached in about 1.5 minutes, the maximum secondary fall to 0.42° below the original level in 6 minutes. The mucous membrane temperature, on the

[6] 0:12, 0:17, etc., indicate the time after the beginning of the experiment; *i.e.*, 0:12 signifies 12 minutes after the beginning of the experiment, 0:17, 17 minutes, etc.



TEXT-FIG. 3.



TEXT-FIG. 4.

TEXT-FIG. 3. Reactions to chilling, rewarming, and amyl nitrite. Temperatures of skin and mucous membrane of faucial tonsil, Experiment 1. *a*, applicator noted to be properly adjusted; *b*, flush of face fading; *c*, breathing shallow; *d*, breathing deeper.

TEXT-FIG. 4. Reactions to chilling, rewarming, and amyl nitrite. Temperatures of skin and mucous membrane of faucial tonsil, Experiment 2. *a*, fan faster; *b*, applicator loose; readjusted; *c*, face flushed; *d*, face fairly white.

other hand, which was already at the high level of 33.86°, fell with amyl nitrite to 33.17°, reached after 6 minutes, and then rose to a level slightly above 34° about 15 minutes after inhalation.

The tonsil here responded to amyl nitrite in the same way as did an acutely inflamed soft palate.[7] The mucous membrane vessels were already dilated; the effect of the relaxing of the mucous membrane blood vessel walls by amyl nitrite must have been overshadowed by the general lowering of blood pressure, the rate of circulation through the mucous membrane vessels was decreased, and the temperature fell. It is possible that increased depth of respiration with amyl nitrite also contributed to the observed fall.

Experiment 2.—Subject 2, L. J. O. June 7, 1919, 10.30 to 11.30 a.m. Mucous membrane thermopile on left tonsil. Skin thermopile on forehead. Respirations 14 per minute. Mouth breathing. Thoracic and abdominal pneumographs. Room temperature 17.12–17.87°C.

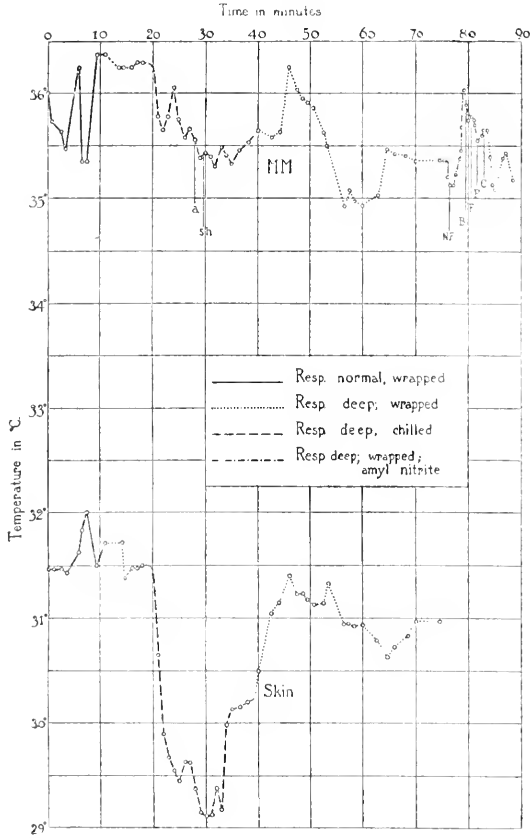
Text-fig. 4 shows the usual depression of skin and mucous membrane temperatures; the latter in this case continued to fall for about 6.5 minutes after cessation of chilling, possibly because of faulty adjustment of the applicator, which at 0:38 was noted to be loose, and was readjusted. The next reading showed a mucous membrane temperature 1.37° above the last reading before chilling. This curve thus probably records a reaction of hyperemia to rewrapping, but it was excluded from the composite graph because of the readjustment of the thermopile. On inhalation of amyl nitrite at 0:49, the response was again a drop in tonsillar temperature—0.94° in 6 minutes; the increase in respiratory amplitude was at most 30 per cent. The increase in respiratory amplitude at 0:4.5 amounted to 84.6 per cent and was followed within 6.5 minutes by a depression of only 1.16°. The temperature fall after amyl nitrite was, then, evidently due in part both to respiratory increase and to general blood pressure fall.

Experiment 3.—Subject 3, S. B. G. June 6, 1919, 5 to 6.30 p.m. Mucous membrane thermopile on left tonsil. Skin thermopile on forehead. Respirations 14 per minute. Mouth open; nostrils plugged. Thoracic and abdominal pneumographs. Room temperature 18.60–20.0°C.

---

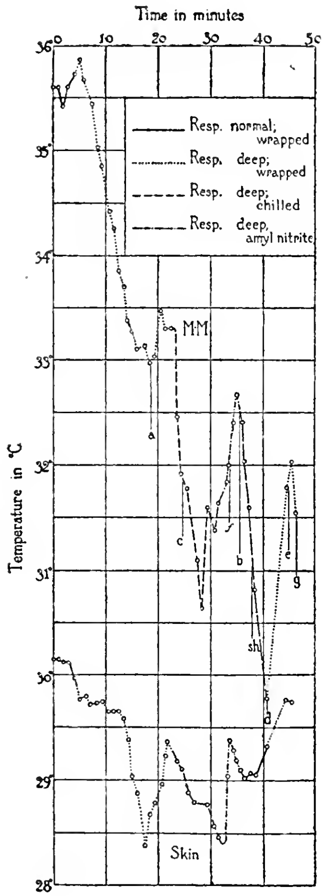
[7] Mudd and Grant,[1] p. 92.

In Text-fig. 5 the chilling and recovery curves proceed normally until 0:46, after which both skin and mucous membrane temperatures fall again, presumably on account of the subject's being inadequately wrapped. This curve is included in the composite although it makes less striking the hyperemia reaction to rewarming. The

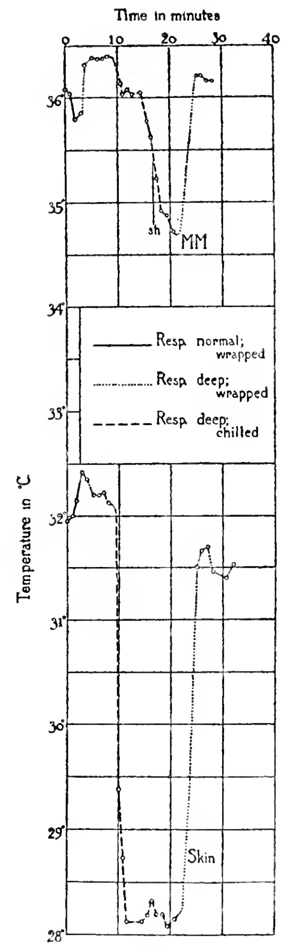


TEXT-FIG. 5. Reactions to chilling, rewarming, and amyl nitrite. Temperatures of skin and mucous membrane of faucial tonsil, Experiment 3. *a*, fan faster; *sh*, starts shivering; *NF*, face not flushed; *B*, second ampule of amyl nitrite inhaled; *F*, face flushed; *P*, still inhaling amyl nitrite; face paler; *C*, stops inhaling.

mucous membrane response to amyl nitrite is here a momentary fall of 0.23° in 1 minute, followed by a sharp rise of 0.90° in 2 minutes, with a more gradual return to normal in about 5 minutes. The predominating factor is evidently the local vasodilation.



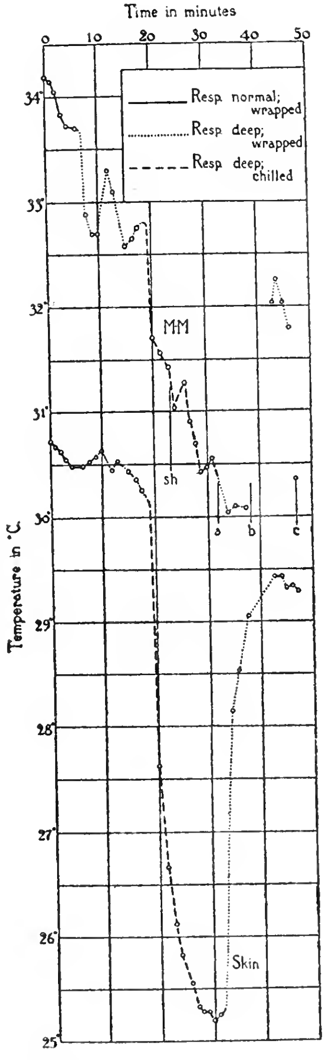
TEXT-FIG. 6.



TEXT-FIG. 7.

TEXT-FIG. 6. Reactions to chilling and amyl nitrite. Temperatures of skin and mucous membrane of faucial tonsil, Experiment 4. *a*, clears throat; *c*, coughed; *f*, face flushed; *b*, flush passing; stops inhaling amyl nitrite; *sh*, is shivering; *d*, clears throat; *e*, respiration too shallow; deepened; *g*, subject fainted.

TEXT-FIG. 7. Reactions to chilling and rewarming. Temperatures of skin and mucous membrane of faucial tonsil, Experiment 5. *sh*, is shivering.



TEXT-FIG. 8. Reaction to chilling. Temperatures of skin and mucous membrane of faucial tonsil, Experiment 6. Tonsil hypertrophic. *sh*, shivers; *a*, applicator holder seemed to be slipping; *b*, applicator off; reapplied; *c*, applicator tip off tonsil.

Experiment 4.—Subject 4, R. L. A. June 9, 1919, 10.56 to 11.46 a.m. Mucous membrane thermopile on left tonsil. Skin thermopile on forehead. Respirations 14 per minute. Mouth open; nostrils plugged. Thoracic and abdominal pneumographs. Room temperature 17.47–18.55°C.

In Text-fig. 6 the mucous membrane depression with chilling is great—3.52° in 18 minutes; the maximum skin fall, on the other hand, is only 0.88°, in 7.5 minutes, and is obliterated after amyl nitrite inhalation. Amyl nitrite, administered at 0:32.5 during chilling, produced a sharp rise in both mucous membrane and skin temperatures—1.02° in 2.5 minutes, and 0.89° in 1 minute, respectively. The recovery curves started up characteristically, but were interrupted by the subject's fainting.

Experiment 5.—Subject 3, S. B. G. June 11, 1919, 4.37 to 5.09 p.m. Mucous membrane thermopile on left tonsil. Skin thermopile on forehead. Respirations 10 per minute. Mouth breathing. Thoracic and abdominal pneumographs. Room temperature 19.65–20.12°C.

Text-fig. 7 shows characteristic chilling curves. Recovery is not quite complete either for skin or mucous membrane, perhaps due to inadequate wrapping. The tonsillar curve was cut short at 0:28 by the slipping of the thermopile applicator.

Experiment 6.—Subject 5, A. G. June 10, 1919, 11.30 a.m. to 12.17 p.m. Mucous membrane thermopile on right tonsil. Skin thermopile on forehead. Respirations 16 per minute. Mouth open; nostrils plugged. Thoracic and abdominal pneumographs. Room temperature 16.90–17.80°C.

The tonsils of this subject had been diagnosed in the Army and in the Washington University as hypertrophic. Text-fig. 8 shows characteristic chilling curves for both tonsil and skin, of large amplitude in each case. The applicator holder seemed to be slipping at 0:31.5, and satisfactory application was not thereafter obtained.

Discussion of Vasomotor Reactions to Chilling.[8]

The difference in recovery of blood supply upon rewarming after chilling exhibited by the palatine tonsils on the one hand, and the palate and pharynx on the other, is of interest, and suggests a corollary

[8] Compare Galeotti, G., *Riforma Med.*, 1920, xxxvi, 205; abstracted in *J. Am. Med. Assn.*, 1920, lxxiv, 1491.

hypothesis. It is well known that persons in robust health and inured to rigorous physical circumstances of living react better after chilling, as for instance by a sea bath, than the less vigorous. It seems possible that one factor in the hardening process of cold bathing, outdoor life, and so forth, with its incident heightened resistance to colds and sore throats, is a training of the vasomotor system in the direction of development of a quick reaction of hyperemia in the pharynx following chilling, such as has been shown to exist in the tonsils of the subjects of the present experiments.

We would reiterate in order to minimize any possibility of misunderstanding. We are concerned in our experiments with excessive chilling of the body surface, which, like overdosage of a useful drug, we believe may have ill effects. Certainly we would not encourage the unreasoning fear of slight drafts and exposure so often encountered. Good ventilation and circulating air in buildings, cold weather, and out-of-door living are needed for vigorous health; many people are unquestionably benefited by cold bathing. But excesses in this direction should also be avoided.

Autoinfections only are under consideration in the present paper. However, it should be clearly borne in mind that colds dependent primarily upon contagion from outside sources are probably of more frequent and widespread occurrence.[9]

Concurrent Bacteriological Studies.

Several instances during the experiments of 1918 seemed at least suggestive of experimental excitation of infection. The case of S. M. (Subject 6) in particular seemed to point to chilling as the exciting factor. The experiment was a blood temperature control in which he sat from 4 to 6.09 p.m., December 4, 1918, with closed mouth, the bulb of a thermometer beneath his tongue. He forced respiration from 4.12 to 6.09 p.m., and was chilled by a fan from 4.47 to 5.09 p.m. Shivering began at 4.48 p.m., and had become very severe by 4.52 p.m. The subject had not been aware of other recent exposure to cold or infection. By the morning of December

[9] For a discussion of this phase of the subject the reader is referred especially to Hill, L., The science of ventilation and open-air treatment. Part 1, *Gt. Britain Med. Research Com., Special Rep. Series, No. 32,* 1919.

5, nasopharyngeal stuffiness had developed sufficiently to cause the remark by a friend that he had a cold. The symptoms persisted 3 or 4 days.[10]

A study of the flora of the nose and throat during the experiments of the summer of 1919 seemed, then, to be well worth while.

Material and Method.—The medium employed was a 5 per cent rabbit blood meat infusion agar. Baked blood agar was also used in each instance as a special medium for *Bacillus influenzæ*. Sputum from each subject was injected into a mouse for typing pneumococcus. Cultures were taken from the nose through the anterior nares, from the tonsil, and from the posterior pharyngeal wall by means of separate swabs. Each swab was immersed in sterile broth and then applied both to a red and baked blood agar plate; the remaining area of the plate was inoculated by means of a platinum loop. Films were made directly from the same swab.

The cultures were incubated for 36 hours. The plates were then divided into eight segments, and every colony in two to four segments of the plate was counted and its nature determined. An attempt was made to count approximately the same total number of colonies daily, in order to detect any marked changes in the flora and particularly in the relative proportions of the bacteria present. On account of the difficulty of differentiating pneumococcus from *Streptococcus viridans* by morphology these two were put in the same group.

Results.—Four different subjects were used in this study. Two developed clinical sore throats; a third had some symptoms of malaise and headache; a fourth was unaffected. The results obtained with the nose cultures were entirely negative. Subjects 5 and 6 showed *Staphylococcus aureus*, Subjects 1 and 3 *Staphylococcus albus*. No attempt was made to sterilize the vestibule of the nose before swabbing. The flora obtained from the nose, as has been found by other investigators[11] was at all times exceedingly sparse, but the above organisms were always found to be present. They showed no changes with exposure. The results obtained from pharynx and tonsil in each instance are given in Tables I to III.

[10] Similarly we have noted what we believe to be an excitation of infection after, and presumably due to chilling in a number of carefully observed instances in our everyday experience.

[11] Thomson, St. C., Diseases of the nose and throat, New York, 1913, 7.

TABLE I.

Subject 6. Bacteriology of Tonsil and Pharynx.

Date.	Place cultured.	*S. viridans* and Pn. II.		*S. hæmolyticus.*		*B. influenzæ.*		Undetermined.		Remarks.
		No. of colonies counted.	Per cent of all colonies.	No. of colonies counted.	Per cent of all colonies.	No. of colonies counted.	Per cent of all colonies.	No. of colonies counted.	Per cent of all colonies.	
1919										
June 4	Left tonsil.	28	93	0	0	2	7	0	0	
	Pharynx.	36	100	0	0	0	0	0	0	
						Present in baked blood.				
" 5	Left tonsil.	43	100	0	0	" "	" "	0	0	
	Pharynx.	31	94	0	0	" "	" "	4	6	
" 6	Left tonsil.	35	100	0	0	" "	" "	0	0	
	Pharynx.	24	86	0	0	" "	" "	4	14	
" 7*	Left tonsil.	52	94	0	0	3	6	0	0	Subject of experiment. Application on left tonsil. Cultures after experiment.
	Pharynx.	40	87	0	0	4	13	0	0	
" 8	Left tonsil.	57	65	13	15	7	8	11	12	Subjective sore throat. Pharynx shows injection with white exudate; tonsillar ring injected.
	Pharynx.	26	56	14	31	6	13	0	0	
" 9	Left tonsil.	35	76	6	13	1	2	4	8	Very slight soreness. Throat no longer injected.
	Pharynx.	34	79	9	21	Present in baked blood.		0	0	
" 11	Left tonsil.	34	94	1	3	1	3	0	0	Throat normal.
	Pharynx.	30	91	3	9	Present in baked blood.		0	0	

* Experiment 7.

TABLE II.

Subject 1. Bacteriology of Tonsil and Pharynx.

Date.	Place cultured.	*S. viridans.*		*S. hæmolyticus.*		*B. influenzæ.*		*S. albus.*		Remarks.
		No. of colonies counted.	Per cent of all colonies.	No. of colonies counted.	Per cent of all colonies.	No. of colonies counted.	Per cent of all colonies.	No. of colonies counted.	Per cent of all colonies.	
1919										
June 10	Left tonsil.	18	42	4	9	9	21	12	28	
	Pharynx.	13	52	0	0	12	48	0	0	
" 11*	Left tonsil.	28	68	3	8	8	20	2	4	Subject of experiment. Application on left tonsil. Cultures after experiment.
	Pharynx.	24	72	0	0	6	18	3	9	
" 12	Left tonsil.	31	55	3	5	5	9	17	31	No after effects.
	Pharynx.	18	54	0	0	12	36	4	9	
" 16†	Left tonsil.	26	53	0	0	6	12	18	35	Subject of experiment. Application on soft palate. Cultures taken before experiment.
	Pharynx.	32	60	0	0	9	17	12	23	
" 17	Left tonsil.	14	45	2	7	8	26	7	22	Subject complains of general malaise; slight headache; no sore throat. Film from swab shows mostly Gram-negative bacilli with a few cocci.
	Pharynx.									
	Red blood.	0	0	0	0		100	0	0	
	Baked blood.	0	0	0	0		100	0	0	
" 19	Left tonsil.	19	56	2	6	5	15	8	24	
	Pharynx.	21	60	0	0	14	40	0	0	
" 20	Left tonsil.	21	50	3	7	7	17	11	26	
	Pharynx.	19	58	0	0	11	33	3	9	

* Experiment 1.

† Experiment 8.

TABLE III.

Subject 5. Bacteriology of Tonsil and Pharynx.

Date.	Place cultured.	*S. viridans.*		*M. catarrhalis.*		*S. aureus.*		*S. albus.*		Remarks.
		No. of colonies counted.	Per cent of all colonies.	No. of colonies counted.	Per cent of all colonies.	No. of colonies counted.	Per cent of all colonies.	No. of colonies counted.	Per cent of all colonies.	
1919										
June 7	Left tonsil.	29	85	0	0	0	0	5	15	
	Pharynx.	21	91	0	0	0	0	2	9	
" 8*	Left tonsil.	49	90	0	0	0	0	5	10	Subject of experiment. Application on left tonsil. Cultures after experiment.
	Pharynx.	27	75	0	0	2	6	7	19	
" 9	Left tonsil.	21	60	3	9	0	0	11	31	No symptoms.
	Pharynx.	31	57	15	27	1	2	7	14	
" 10†	Left tonsil.	27	52	4	8	2	4	19	36	Subject of experiment. Application on right tonsil. Cultures after experiment.
	Pharynx.	34	81	0	0	3	7	5	12	
" 11	Left tonsil.	34	85	1	2	0	0	5	13	
	Pharynx.	39	93	0	0	0	0	3	7	
" 12	Right tonsil.	30	73	0	0	4	10	7	17	
	Pharynx.	34	87	2	5	1	3	2	5	
" 13‡	Right tonsil.	40	88	2	5	0	0	3	7	Subject of experiment. Application on pharynx. Cultures after experiment.
	Pharynx.	27	70	7	19	0	0	4	11	
" 14	Right tonsil.	21	58	12	34	0	0	3	8	
	Pharynx.	15	45	4	11	11	39	2	5	
" 15										No cultures. Subject has soreness on swallowing.

June 16§	Right tonsil.	21	52	11	27	0	0	8	20	Subject of experiment. Application on pharynx. Marked congestion of tonsillar ring and pharynx.
	Pharynx.	29	60	15	31	0	0	4	9	
" 17	Right tonsil.	34	72	3	7	8	17	2	4	Throat injection milder. No soreness on swallowing.
	Pharynx.	27	75	6	16	3	8	0	0	
" 18‖	Right tonsil.	25	59	2	5	15	36	0	0	Subject of experiment. Application on soft palate. Cultures after experiment.
	Pharynx.	31	77	4	10	5	13	0	0	
" 19	Right tonsil.	28	54	15	28	9	18	0	0	Soreness on swallowing. Injection of tonsillar ring.
	Pharynx.	32	64	11	22	7	14	0	0	
" 20										No cultures. Sore throat disappeared.
" 21	Right tonsil.	35	83	0	0	4	10	3	7	No soreness. No injection.
	Pharynx.	31	93	1	3	0	0	1	3	
" 23	Right tonsil.	31	81	2	5	1	2	4	11	" " " "
	Pharynx.	27	81	1	3	2	6	3	9	

* Experiment 9.
† Experiment 6.
‡ Experiment 10.
§ Experiment 11.
‖ Experiment 12.

From the pharynx and tonsil of Subject 6 before the experiment there were cultured *Streptococcus viridans*, Pneumococcus Type II atypical, and *Bacillus influenzæ*. He was then subject of Experiment 7. Within 24 hours he had a clinical sore throat; coincident with this, there was a sudden appearance of *Streptococcus hæmolyticus* in the cultures from both tonsil and pharynx. During the days following, with the disappearance of the sore throat, the number of colonies of *Streptococcus hæmolyticus* fell off rapidly. The remaining bacteria showed no evident change.

Experiment 7.—Subject 6, S. M. June 7, 1919, 3.45 to 6 p.m. Mucous membrane thermopile on left tonsil. Respirations 14 per minute. Mouth open; nostrils plugged. Room temperature 18.10–19.60°C. 0:00 to 0:31.5. Wrapped; normal breathing; swallowed many times. 0:31.5 to 0:44. Wrapped; deep breathing. 0:44 to 0:55.5. Unwrapped; fan on back; deep breathing. 0:52. Shivers. 0:55.5. Coughs, chokes, applicator removed; blood flecks seen about terminals. 4.57 p.m. Experiment started again. 0:00 to 0:06. Wrapped; deep breathing. 0:06 to 0:13.5. Unwrapped; fan on; deep breathing; shivering; swallows many times. 0:13.5 to 0:33.5. Wrapped; deep breathing. 0:33.5. Conditions the same; amyl nitrite inhaled. 0:35 to 0:44. Wrapped; deep breathing; some coughing and swallowing. 0:44 to 0:53. Hot water bag around subject; wrapped; deep breathing.

On the following morning the feeling of soreness had practically left the traumatized tonsil, but the posterior wall of the oropharynx felt sore. On inspection a localized area of injection bearing a whitish exudate was seen on the posterior wall of the oropharynx. In culturing, the contact of the swab on this area was a little painful, and the culture yielded streptococci, as explained above. The tonsillar ring was injected, but the feeling of soreness on the tonsil entirely passed off during the day and contact of the swab in culturing was hardly felt. Thus the traumatized tonsil showed less evidence of being the site of an active infection than the posterior pharyngeal wall, which was thought not to have been directly traumatized, but of course we cannot exclude the possibility that the oropharynx was infected by hemolytic streptococci from the tonsillar crypts—so commonly a habitat for them—which were missed in the earlier cultures and were disseminated by the experimental trauma and swallowing.

In Subject 1 there were present *Streptococcus viridans*, *Bacillus influenzæ*, *Streptococcus hæmolyticus*, and *Staphylococcus albus*. Following Experiment 1 there were no noteworthy changes. The plate inoculated from the pharynx about 26 hours after Experiment 8, however, showed a pure culture of *Bacillus influenzæ;* the tonsillar

plate showed also a slight relative increase. The film from the pharynx showed practically all Gram-negative bacilli with an occasional coccus. The subject had no sore throat, but complained of general malaise, slight headache, and some chilly sensations.[12] The pharynx culture made 96 hours after Experiment 8 was practically the same as before the experiment, *Streptococcus viridans* and *Staphylococcus albus* appearing as before.

Experiment 8.[13]—Subject 1, W. G. E. June 16, 1919, 3.20 to 4.07 p.m. Mucous membrane thermopile on anterior half of soft palate, left side. Respirations 16 per minute. Mouth open; nostrils plugged. Room temperature 20.22–20.90°C. 0:00 to 0:05.5. Wrapped; normal breathing. 0:05.5 to 0:11. Wrapped; deep breathing. 0:11 to 0:32. Unwrapped; fan on back; deep breathing. 0:32 to 0:47. Wrapped; deep breathing.

With the applicator resting against the anterior soft palate there is no reason for supposing trauma to the tonsils and pharynx. Organisms could hardly have been introduced from outside by the applicator, for this, with the thermopile terminals attached, was freshly coated with shellac in alcoholic solution before being adjusted for an experiment.

Subject 5 was the subject of five experiments, June 8, 10, 13, 16, and 18. On June 15 he noted a soreness on swallowing, and on June 16 the entire posterior pharynx and tonsillar ring were distinctly injected. The experiment of June 16 produced no sudden increase in symptoms; on June 17 the sore throat had practically disappeared. On June 19, 36 hours following an experiment, the subject again developed a soreness on swallowing, with congestion of the posterior pharynx and tonsillar ring. On June 20 the symptoms had subsided. On June 21 the wares no longer any injection or soreness.

This subject had present in his throat *Streptococcus viridans*, *Staphylococcus aureus*, and *Staphylococcus albus*. 24 hours following the first experiment there was noted for the first time the appearance of *Micrococcus catarrhalis*. Subsequently there appeared to be a certain degree of correlation between the appearances of sore throat and the rises in relative numbers of *Micrococcus catarrhalis* colonies. No other change in the bacterial flora was apparent.

[12] Compare the effect of infecting monkeys with *B. influenzæ* (Blake, F. G., and Cecil, R. L., *J. Am. Med. Assn.*, 1920, lxxiv, 170).

[13] For Experiment 1 see p. 93.

Experiment 9.—Subject 5, A. G. June 8, 1919, 3.38 to 5.06 p.m. Mucous membrane applicator on left tonsil. Respirations 10 per minute. Mouth open; nostrils plugged. Room temperature 18.1–19.45°C. 0:00 to 0:06. Wrapped; normal breathing. 0:06 to 0:15. Wrapped; deep breathing. 0:15 to 0:43. Unwrapped; fan on back; deep breathing; some swallowing, coughing, and clearing of throat; after 0:17, shivering. 0:43 to 1:20. Wrapped; deep respiration. 1:07. Inhales amyl nitrite. 1:20 to 1:28. Hot water pad to back; deep respiration.

The possibility that trauma to the tonsil was responsible for the appearance of *Micrococcus catarrhalis* after this experiment cannot be excluded.

Experiment 10.[14]—Subject 5, A. G. June 13, 1919, 3 to 3.46 p.m. Mucous membrane applicator on posterior wall of oropharynx. Respirations 16 per minute. Mouth open; nostrils plugged. Room temperature 18.45–19.05°C. 0:00 to 0:04.5. Wrapped; normal breathing. 0:04.5 to 0:15. Wrapped; deep breathing. 0:15 to 0:26. Unwrapped; fan on back; deep breathing; coughed; cleared throat; contraction of pharyngeal muscles; after 0:22, shivers. 0:26 to 0:46. Wrapped; deep breathing; coughed and cleared throat several times; pharynx appeared normal.

Experiment 11.—Subject 5, A. G. June 16, 1919, 10.30 to 11.30 a.m. Mucous membrane applicator on posterior pharyngeal wall. Respirations 14 per minute. Mouth open; nostrils plugged. Room temperature 18.90–19.80°C. 0:00 to 0:04.5. Wrapped; normal breathing; contractions of pharyngeal muscles. 0:04.5 to 0:23.5. Wrapped; deep breathing. 0:23.5 to 0:32. Unwrapped; fan on back; deep breathing. 0:32 to 1:00. Wrapped; deep breathing; contractions of pharyngeal muscles.

Experiment 12.—Subject 5, A. G. June 18, 1919, 10.20 to 11.12 a.m. Mucous membrane applicator on soft palate, middle part. Respirations 14 per minute. Mouth open; nostrils plugged. Room temperature 18.75–19.55°C. 0:00 to 0:05.5. Wrapped; normal breathing. 0:05.5 to 0:15.5. Wrapped; deep breathing. 0:15.5 to 0:27. Unwrapped; fan on back; deep breathing. 0:27 to 0:52. Wrapped; deep breathing.

In Subject 3 there were present in the throat *Streptococcus viridans*, Pneumococcus Type IV, and *Staphylococcus albus*. Cultures were made daily from June 4 to 19; he was the subject of an experiment on June 6, 9, 14, and 17. There was practically no change in the bacterial flora of this subject throughout the entire period studied. Neither did subjective or objective signs of sore throat or cold develop. Frequently the cultures from the pharynx were almost sterile, there being from three to ten colonies over the entire plate.

[14] For Experiment 6 see p. 99.

Discussion of Bacteriological Results.

Streptococcus viridans was found in all four of the individuals studied, *Bacillus influenzæ* in two, pneumococcus in two, and *Micrococcus catarrhalis* in one subject.

In Subject 6 the increased number of *Streptococcus hæmolyticus* was definitely synchronous with the presence of a sore throat. There appears to have been a correlation between the high *Micrococcus catarrhalis* counts and the presence of sore throats in Subject 5. The pure culture of *Bacillus influenzæ* in the pharynx of Subject 1 was not coincident with sore throat but with malaise, slight chilliness, and headache.

These results in no sense prove, however, that the sore throats were caused by the increased number of bacteria cultured from the mucous membranes, or that the apparent increase of microorganisms was caused by the ischemia of the mucous membranes incident upon chilling of the body surface. The method is subject to so many sources of error, and the amount of data thus far obtained is so small, that we do not feel justified in drawing any conclusions. To attribute the apparent proliferation of pathogenic microorganisms to the effect of chilling would seem to be in harmony with the great wealth of clinical and common observation which points to excessive chilling, under proper circumstances, as an efficient excitant of infection of the mucous membranes by their indigenous pathogenic bacteria. Although it is possible that the apparent proliferation was due to the local ischemia incident upon chilling, the inaccuracy of the bacteriological method and the insufficient data make it impossible to assume that this is so. The effect of trauma by the thermopiles, the possibility of transient changes in the flora of the mucous membranes caused by swallowing, gagging, or other muscular activity in the pharynx pressing a plug of bacteria from the tonsillar crypts, the fact that the subject's mouth was held open throughout the experiments, with the accompanying accumulation of mucus on the membranes, the errors necessarily introduced in each stage of making the cultures, and the inaccuracy of any method depending upon swab cultures, all tend to confuse the results. We present the data given above as a contribution to the etiology of upper respiratory infections, and not with the idea that the study is in any sense complete in itself.

TABLE IV.

Observations on Concentration and Reaction of Urine.

Experiment No.	Increase in respiration.	Deep respiration.	Deep respiration; chilled.	Deep respiration.	Reaction to litmus.		Color.		Clarity.		Decrease in acidity in amount of 0.1 N NaOH neutralized by 10 cc. of urine.
					Before experiment.	After experiment.	Before experiment.	After experiment.	Before experiment.	After experiment.	
	per cent	*min.*	*min.*	*min.*							*cc.*
9*	77.9	9	28	45					Clear.	Cloud of phosphates.	
4*	88.9	18.5	17.5	6		Neutral.	Dark yellow.	Light yellow.			
13*		17.5	19.5	21	Acid.	Acid.	Yellow.	Very slightly lighter.			
6	75.4	12	13.5	15	"	Alkaline.	"	Light yellow.	Clear.	Phosphates.	4.25 (titrated next day).
1	42.7	6	5.5	42.5	"	"	"	" "	"		1.76
14 } 15 }	45.4	11.75	22	17	"	Neutral to alkaline.	"	Slightly lighter yellow.	"	Cloudy.	1.24
10	69.2	10.5	11	20	"	Alkaline.					2.54
16	54	7.5	22	20	"	"					2.16
11	30.1	19	8.5	28	"	"					4.90
8	30.1	5	21	15.5	"	Acid.					0.00
17	44.6	6	16	19.5	"	"					2.28
12	52.3	10	11.5	28	Neutral.	Alkaline.					0.95
Average.	49.3	9.75	14.56	22.83							2.23

* Not included in average.

Observations on Urine.

It was thought that the chilling in the experiments might produce sufficient renal congestion to give albuminuria. This, however, was not the case. In fourteen experiments in which urine specimens were taken before and after the experiment the results were in all instances negative. The room temperature in these experiments varied from 16.9–20.9°C.; the time of exposure, unwrapped, to the draft of the electric fan was from 5.5 to 28 minutes, the average 16.75 minutes. Severe shivering was often produced. In twelve of the experiments urinalysis for sugar was done, also with negative results in all.

In all of five experiments in which the color was noted it was lighter after than before the experiment, denoting the secretion of a more dilute urine during the experiment.

The most interesting change in the urine produced by the experimental conditions was a decrease in its acidity. Specimens were titrated with phenolphthalein against 0.1 N sodium hydroxide in nine experiments; in eight a decrease in acidity after the experiment was noted, amounting to the equivalent of from 0.95 to 4.90 cc. of 0.1 N sodium hydroxide per 10 cc. of urine; the average decrease was 2.23 cc. This lowered titer was doubtless due in part to dilution of the urine, but in part also represented a true fall in hydrogen ion concentration, as is shown by the fact that a change in the urinary reaction demonstrable with litmus paper was noted in seven out of ten experiments. The mechanism of the experimental alteration in the reaction of the urine has been further analyzed by Grant and Goldman;[15] the decreased acidity has been found to be referable to the forced respiration rather than to the chilling.

The results of the observations on concentration and reaction of urine are given in Table IV.

SUMMARY.

Improvements are described in the method of following temperature changes, and thus alterations in vasomotor tone, in exposed mucous membranes. Invention of an applicator holder, by means of which

[15] Unpublished investigation.

more sure and stable apposition of the thermopile terminals to the mucous surface may be effected has been the chief advance. Minor improvements have been the use of a saliva ejector and of better calibration technique.

The palatine tonsils, like the palate, pharynx, and skin, react to chilling of the body surface with reflex vasoconstriction and ischemia. On rewarming the subject the tonsils quickly more than recover their former blood supply, actually becoming hyperemic; the skin returns to about its control condition; the pharynx and palate remain somewhat ischemic.

The hypothesis is advanced that one factor in the beneficial hardening effect of cold bathing and outdoor living, with its incident heightened immunity to respiratory infection, may be the training of the vasomotor system in the direction of development in the pharynx of a reaction of hyperemia following chilling, similar to that observed in the tonsils of the present subjects.

With inhalation of amyl nitrite, the skin temperature has always shown a sharp transient rise. The mucous membrane, if relatively ischemic, responds by a rise corresponding to the skin flush. If already hyperemic, local vasodilation in the mucous membrane with amyl nitrite is more than counterbalanced by the lowering of the general blood pressure, and the temperature falls.

The flora of the pharynx and tonsils, studied by the unsatisfactory method described, showed, in several instances, after experimentation changes apparently due to proliferation of one of the microorganisms already present. In one case *Streptococcus hæmolyticus*, in one *Micrococcus catarrhalis*, and in a third *Bacillus influenzæ* was the organism showing a relative increase in numbers. The first two instances were associated with sore throat, the third with slight constitutional symptoms.

The chilling in the experiments in no instance produced albuminuria or glycosuria, although a more dilute urine was apparently excreted during the experiments. A fall in hydrogen ion concentration, referable to the forced respiration, was noted.

It is a pleasure to thank Professor Joseph Erlanger of the Department of Physiology for his many courtesies.

HOMEOTRANSPLANTATION AND AUTOTRANSPLANTATION OF THE SPLEEN IN RABBITS.

III. Further Data on Growth, Permanence, Effect of Age, and Partial or Complete Removal of the Spleen.

By DAVID MARINE, M.D., and O. T. MANLEY, M.D.

(*From the Department of Experimental Medicine of Western Reserve University, Cleveland.*)

(Received for publication, February 27, 1920.)

We have been unable to find any references in the literature to the transplantation of fragments of spleen to parts of the body widely separated from the normal neurovascular field of this organ in addition to those referred to in 1917. At that time[1,2] we reviewed the literature and reported our first experiments with spleen homeografts and autografts in fifteen rabbits. The present paper includes the data of further experiments with homeografts and with autografts, together with certain general physiological reactions relative to the spleen which this study has emphasized.

Method.

The method consists of transferring small fragments of the spleen roughly 2 by 2 mm. to the subcutaneous tissues of the abdomen under strict aseptic precautions. The tissue to be transplanted was kept in isotonic salt solution at temperatures varying between 36° and 39°C. Sometimes the tissue was transplanted within a few minutes after removal, while in others it was kept in salt solution as long as 3 hours. The fragment to be transplanted was rinsed in the salt solution, but no other attempt to remove contained blood was made.

[1] Manley, O. T., and Marine, D., The transplantation of splenic tissue into the subcutaneous fascia of the abdomen in rabbits, *J. Exp. Med.*, 1917, xxv, 619.

[2] Marine, D., and Manley, O. T., Influence of age on the permanence of subcutaneous autografts of the spleen in rabbits, *Proc. Soc. Exp. Biol. and Med.*, 1916–17, xiv, 123.

After making a transverse abdominal skin incision approximately 2 cm. in length, usually one on each side of the umbilicus, the subcutaneous fascia was lifted with fine forceps and an area free from blood vessels was punctured with a cataract knife, the tissue introduced, and the fascial opening closed with a ligature. The skin incision was sutured and sealed with celloidin. All operative procedures were carried out under ether anesthesia. All subsequent examinations were direct (autopsy or operation). If the examination was made by operation, ether anesthesia was used, and with aseptic precautions the skin was incised, the graft or its site was exposed, measurements were taken, the graft was removed for microscopic examination or left *in situ*, and the skin closed with suture and sealed with celloidin. The intervals between examinations are given in the tables. This technique has been employed in all our transplantation work with ductless gland tissues.

Both homeografts and autografts were studied in relation to taking and growth or regeneration, in relation to age of the animals, and in relation to partial and complete removal of the spleen and to permanence of the grafts.

Homeotransplantation.

The data of the experiments are given in Table I. Only the rabbits which had not been subjected to previous homeografts of any tissue are included in the table. This was done to avoid the untoward effect of active immunity which such grafts produce and to render conditions as favorable as possible for taking and growth. Notwithstanding this important precaution not a single graft was found active after the 30 day period, a period arbitrarily chosen, but somewhat longer than the time necessary for the full development and destructive effect of the active immunity. The experiments were not studied from the standpoint of determining how rapidly the grafts were destroyed, though by comparison they show very clearly that the destruction of spleen is much more rapid than that of thyroid tissue transplanted under the same conditions. Thus in a large number of routine homeotransplants of thyroid we have obtained about 10 per cent active at the end of 30 days, though ultimately (in the course of

60 to 90 days) these also might be destroyed. These observations indicate that spleen tissue produces a high degree of immunity more quickly than does thyroid.

The well established favorable factor of blood relation in the transplantation of certain tissues was also utilized in the hope that grafts might survive beyond the 30 day period, but with spleen we were unsuccessful. The antigenic power of spleen, then, is very high and on a level with that of other hematopoietic tissues. In a few instances in which the grafts were examined at the 7th, 8th, and up to the 16th day, active splenic tissue, well established blood supply, and evidence of growth were found. This is observed generally with homeografted tissues. Many literature reports of positive homeografts of tissues are misleading because they have emphasized or reported only this early stage. Effective immunity to foreign proteins in general does not become manifest until the 8th to 10th day, as shown by studies on anaphylaxis. Within this period homeografts usually take and often show growth, while after this period rapid destruction takes place. Different tissues show different rates of destruction. In our experience lymphoid (lymph gland and thymus) and splenic tissues undergo destruction more rapidly than thyroid, and thyroid cells more rapidly than cells rich in lipoids, as adrenal cortex and interstitial cells of the ovary and testis. Associated with this destruction there is the well known infiltration or invasion of the graft with lymphoid cells. Much importance has been assigned to this phenomenon and some authors, notably Murphy and his coworkers,[3] have claimed for it a primary importance in the death and destruction of homeografts. We cannot subscribe to this view. We believe that the primary injury is due to the action of antibodies and that the lymphoid cells are attracted to the site secondarily, and in response to a special low grade irritation which injured cells set up. Loeb[4] and Sittenfield[5] also have expressed the view that lymphocytes are not primarily concerned in the destruction of foreign proteins.

[3] Murphy, Jas. B., and Morton, J. J., The lymphocyte in natural and induced resistance to transplanted cancer. II, *J. Exp. Med.*, 1915, xxii, 204.

[4] Loeb, Leo, Multiple transplantations of the thyroid and the lymphocytic reaction, *J. Med. Research*, 1918–19, xxxix, 71.

[5] Sittenfield, M. J., The significance of the lymphocyte in immunity to cancer, *J. Cancer Research*, 1917, ii, 151.

TABLE I.

Homeotransplantation of Spleen.

Rabbit No.	Series No.	Age.	Sex.	Date. Splenectomy.	Date transplanted. Spleen used.		First examination.	Final examination.	Additional data.
					R.*	L.			
		days		*1917*	*1917*	*1917*			
1	4–46	26	M.	Jan. 18 Complete.		Jan. 18 3–155		L. 54–	Jan. 18. L. adrenalectomy. Thyroids and thymus enlarged.
2	4–48	33	F.	Jan. 25 Complete.		Jan. 25 3–157	L. 47±†	" 132–	Thymus atrophic. Jan. 25. L. adrenalectomy; homeograft of adrenal.
3	4–52	40	M.	Feb. 1 Complete.	Feb. 1 3–159		R. 40–	R. 125–	Feb. 1. L. adrenalectomy. Thymus present; thyroid large.
4	4–54	47	"	Jan. 30 Complete.	Jan. 30 3–161			" 42–	Jan. 30. L. adrenalectomy; homeograft of adrenal. Thymus large; thyroid normal.
5	4–64	54	F.	Feb. 6 Complete.	Feb. 6 3–163		R. 35–	" 121–	Feb. 6. L. adrenalectomy. Thymus atrophic; thyroid normal.
6	4–74	61	"	Feb. 13 Complete.	Feb. 13 3–165		" 58–	" 114–	Feb. 13. L. adrenalectomy; homeograft of adrenal. Thymus large; thyroid normal.
7	4–94	90	M.	Mar. 22 Complete.		June 7 3–184		L. 98–	Mar. 22. L. adrenalectomy. Thymus cellular; thyroid normal.
8	4–17	123	"	*1915* Dec. 3 Complete.		*1915* Dec. 16 1–320		" 67–	Spleen was enlarged; thymus small; adrenals small. Dec. 3. Thyroidectomy.
9	4–78	168	"	*1917* Feb. 15 Complete.		*1917* Feb. 15 3–169		" 56–	Feb. 15. L. adrenalectomy. Chronic nephritis; thymus atrophic; thyroid and parathyroids enlarged.

10	4-2	Adult.	M.	*1915* Aug. 26 Complete.		*1915* Nov. 29 1-313		L. 15−	Nov. 29. Thyroidectomy.
11	4-3	"	F.	Aug. 26 Complete.		Dec. 16 1-320		" 65−	" 29. "
12	4-4	"	M.	Nov. 5 Complete.	*1915* Dec. 16 1-320	Nov. 29 1-313		R. 67− L. 17−	" 29. "
13	4-5	"	"	Nov. 5 Complete.	Dec. 18 1-321			R. 63−	Dec. 2. "
14	4-6	"	F.	May 11 Complete.		Dec. 2 1-315 Dec. 16 1-320		L. 14±(Dec. 2). " 65−(" 16).	" 2. "
15	4-7	"	M.	Nov. 5 Complete.		Dec. 16 1-320		L. 65−	" 2. "
16	4-8	"	"	Nov. 5 Complete.		Dec. 16 1-320		" 67−	" 3. "
17	4-9	"	F.	Nov. 5 Complete.		Dec. 16 1-320		" 119−	" 3. "
18	4-42	"	"	*1916* Dec. 12 Complete.		*1916* Dec. 12 3-151		" 43−	" 12. " L. adrenalectomy.
19	4-44	Old adult.	M.	Dec. 19 Complete.	*1917* Feb. 20 1-411			R. 23−	Dec. 12, 1916. Thyroidectomy; L. adrenalectomy.
20	4-93	52	F.	*1917* Mar. 20 ½ cm. Apr. 17 1 cm.		*1917* Apr. 17 3-179		L. 52−	Mar. 20. L. adrenalectomy. Thymus atrophic; thyroid normal.

* R. indicates right; L., left.

† The condition of the transplants at the different examinations is expressed by plus or minus signs instead of the actual measurements obtained in order to facilitate comparison. The figures express the intervals in days.

TABLE I—*Continued.*

Rabbit No.	Series No.	Age.	Sex.	Date. Splenectomy.	Date transplanted. Spleen used. R.*	Date transplanted. Spleen used. L.	First examination.	Final examination.	Additional data.
		days		*1917*	*1917*	*1917*			
21	4-104	80	M.	Apr. 17 ½ cm.		Apr. 17 3-179	L. 53−	L. 163−	All organs intact; same litter as Nos. 4-105 and 4-106; thyroid slightly enlarged; thymus present.
22	4-105	80	"	Apr. 17 1 cm.		Apr. 17 3-179		" 53−	All organs intact.
23	4-106	80	"	Apr. 17 1 cm.		Apr. 17 3-179	L. 53−	" 163−	" " " thymus small.
24	4-108	80	"	Apr. 17 ⅔ cm.		Apr. 17 3-192		" 52−	" " "
25	4-98	104	F.	Apr. 10 ½ of spleen.		Apr. 10 3-177	L. 58±	" 169−	Thyroid lobes large, vascular; thymus normal.
26	4-101	60	M.		Apr. 10 3-177			R. 56−	June 26, 1916. R. adrenalectomy. Oct. 10. Gonadectomy. Mar. 13, 1917. Thyroidectomy. Spleen small; thymus very large.
		mos.							
27	4-65	1	"			Feb. 6 3-163		L. 23−	Feb. 6. Thyroidectomy; much enlarged. H_2SO_4.
28	4-49	4	F.			Jan. 25 3-158		" 21−	Parathyroids very large; thymus large. Jan. 25. Thyroidectomy; H_2SO_4.
29	4-50	1	"			Jan. 25 3-157		" 40−	" 25. " H_2SO_4. Spleen small; parathyroids very large.
30	4-55	1	"		Jan. 30 3-162		R. 14++	R. 35−	Jan. 30. Thyroidectomy; H_2SO_4. Spleen enlarged; parathyroids very active.

31	4-56	4	F.		Jan. 30 3-162			R. 30−	Jan. 30. Thyroidectomy; H_2SO_4. Spleen normal; thymus active.
32	4-57	4	"		Jan. 30 3-162			" 30−	Jan. 30. Thyroidectomy; H_2SO_4. Adrenals and spleen normal; thymus atrophic.
33	4-58	4	M.		Jan. 30 3-162			" 30−	Jan. 30. Thyroidectomy; KOH.
34	4-59	4	"		Feb. 1 3-159			" 28−	Feb. 1. " H_2SO_4. Spleen and thymus normal.
35	4-60	4	"		Feb. 6 3-159			" 28−	Feb. 6. Thyroidectomy; H_2SO_4 and KOH. Spleen small; thymus very large.
36	4-61	4	F.			Feb. 6 3-163		L. 23+	Feb. 6. Thyroidectomy; H_2SO_4. Thymus atrophic.
37	4-76	5	M.			Feb. 13 3-166	L. 16+	" 42−	Feb. 13. Thyroidectomy; KOH.
38	4-62	5	F.			Feb. 6 3-163		" 23−	" 6. " KOH.
39	4-75	Adult.	"			Feb. 13 3-165	L. 16+	" 42−	" 13. " H_2SO_4 and KOH.
40	4-81	"	"			Feb. 15 3-169		" 8+	Jan. 23. Thyroidectomy.
41	4-82	"	"		Feb. 15 3-174			R. 42−	" 23. "
42	4-99	"	M.		Apr. 10 3-177			" 7+	Sept. 28, 1916. R. adrenalectomy. Jan. 16, 1917. L. adrenalectomy. Mar. 3. Thyroidectomy; H_2SO_4. Thymus atrophic; spleen small.
43	4-100	"	"		Apr. 10 3-177			" 58−	Sept. 22, 1916. R. adrenalectomy. Jan. 11, 1917. L. adrenalectomy. Mar. 13. Thyroidectomy. Thymus atrophic; spleen small.

TABLE I—*Concluded.*

Rabbit No.	Series No.	Age.	Sex.	Date. Splenectomy.	Date transplanted. Spleen used. R.*	Date transplanted. Spleen used. L.	First examination	Final examination.	Additional data.
					1917				
44	4-102	Adult.	M.		Apr. 10 3-177			R. 7+	May 25, 1916. R. adrenalectomy. June 1. L. adrenalectomy. Dec. 7. Thyroidectomy; gonadectomy. Spleen small; thymus atrophic.
45	4-109	"	"		Apr. 10 3-177			" 58−	Sept. 26, 1916. R. adrenalectomy. Jan. 11, 1917. L. adrenalectomy. Mar. 13. Thyroidectomy. Thymus large; spleen normal; L. adrenal much enlarged.
46	4-40	"	"		Jan. 25 3-158			" 26−	Dec. 26, 1916. Thyroidectomy; H_2SO_4.
47	4-21	"	"			*1916* Dec. 19 3-154		L. 34−	Mar. 11, 1916. R. adrenalectomy. Dec. 19. L. adrenalectomy. Jan. 9, 1917. Gonadectomy. Thyroid normal; thymus very large.
48	4-39	"	"		Jan. 25 3-158			R. 26−	Jan. 2. Thyroidectomy.
49	4-43	"	"		Jan. 25 3-158			" 26−	" 2. " Previous homeografts.
50	4-87	Old adult.	F.			*1917* Feb. 27 3-167		L. 23−	Jan. 22. Thyroidectomy. Many previous homeografts.

Effect of Age.—This factor has no appreciable influence. In this series rabbits of known age and parentage, varying from 26 to 168 days, as well as a large number of adults of different but known ages, were used. In no instance did the graft survive the 30 day period. Variations in the rate of destruction depending on age must be expected, but their detection in the case of spleen would require frequent examinations within the 30 day period. Further attempts were made to determine whether splenectomy, partial or complete, had any effect. No noteworthy difference in the outcome even in young rabbits was detected whether the spleen was intact, or partially or completely removed at the time of, before, or after transplantation. The experiments do not eliminate the possibility of an advantage accruing to the transplant from induced splenic insufficiency, but indicate that if splenectomy is of aid it must be looked for by frequent examinations within the first 30 days, since by that time the developed immunity has destroyed any possible evidence.

Thyroidectomy and partial or complete adrenalectomy combined or separate likewise have no noteworthy effect on delaying destruction of the grafts and, therefore, probably on the degree of immunity developed. In this connection it may be pointed out that Gates[6] was unable to detect any noteworthy difference in the degree of immunity to sheep erythrocytes and typhoid bacilli obtained in guinea pigs with and without partial removal of the adrenal glands. We have found[7] that thyroidectomy and splenectomy alone or combined have no marked effect on the antibody formation following injection of sheep erythrocytes in rabbits. Bullock and Rohdenburg[8] find that splenectomy has no influence on the immunity to transplanted tumors.

[6] Gates, F. L., Antibody production after partial adrenalectomy in guinea pigs, *J. Exp. Med.*, 1918, xxvii, 725.

[7] Unpublished results.

[8] Bullock, F. D., and Rohdenburg, G. L., Splenectomy exerts no appreciable influence upon immunity against transplanted tumors, *J. Cancer Research*, 1917, ii, 465.

Autotransplantation.

The data concerning these experiments are given in Tables II and III. Usually two fragments were transplanted, one to the right and the other to the left of the umbilicus, and designated as right and left transplants. In the six experiments reported in 1917 one failed and in the present series one failed. This was due to infection. Failure of autografts to take always indicates some gross technical error. Some of these rabbits had been used for homeotransplantation of other tissues—adrenal, thyroid, and sex glands. Previous or coincident homeografts have no demonstrable effect on the taking or growth of autografts. The subsequent course of these autografts was followed and it was found to be modified by several factors, the most striking of which are age and partial or total removal of the spleen.

Effect of Age.—The experiments have been arranged in Tables II and III according to the age at the time of transplantation. The youngest rabbit used was 26 days old and the oldest whose age was definitely known was 320 days; one other is listed as 586+ days because it had been in the laboratory for this length of time before being utilized for this purpose. A maximum of three examinations is given in the tables, although in several instances five, six, and more examinations have been made. A consideration of the series as a whole shows a distinct decrease in the amount of growth of the transplants as one passes from the youngest to the oldest rabbits of the series, although other factors remained as constant as possible. The greatest and most rapid growth of transplants occurred in the youngest rabbits, also the least growth in the oldest. While this decrease with age in the rate and amount of growth is gradual, the differences become striking about the 4th to 5th month. In our series the first instance of failure to obtain marked growth was in a rabbit 132 days old. The remaining ten rabbits, 167 or more days old at the time of transplantation, failed to show marked growth. This change in the rate of growth corresponds roughly with the time of sexual maturity or early adult life. There is a suggestion that in adult rabbits transplants that in the 1st month showed some growth tended to involute or undergo atrophy later. This tendency to atrophy or

involution was not seen in young rabbits with transplants of similar duration. As suggested in a previous note[2] two factors (possibly others) may be considered. The first is that growth of the transplants is a part of the normal growth of the animal and ceases when physical growth is complete. This possibility is disproved by the fact that such transplants fail to show marked growth even in the young if the spleen is not removed. The second factor would presuppose that the value of the spleen to the organism decreases with age and that after adult life whatever functions the organ normally has may be assumed by other tissues, possibly lymph glands and bone marrow. This view is supported by the results obtained by the removal of the spleen which follow.

Effect of Partial and Complete Removal of the Spleen on Autografts.—The data of these fifteen experiments are given in Table III. The amount of spleen removed and the dates are given in the table. Usually less than 0.5 cm. of the anterior portion was removed at the time of transplantation.[9] The youngest rabbit with partial removal of spleen and transplantation was 52 days old and the oldest 124 days. The same differences in the growth of transplants, dependent upon age and independent of amount of spleen removed, are noted as in Table II. Thus in No. 1, a 52 day old rabbit in which one-fourth of the spleen was removed, the transplants were ++ at 80 days and +++ at 177 days, while in No. 15, a 124 day old rabbit in which one-eighth of the spleen was removed, no growth took place in 132 days. Between these extremes there is the same evidence of gradation of growth dependent upon age as shown in Table II. There is no instance of a very young rabbit with a minimum amount of spleen removed. Such experiments would show whether in the absence of splenic deficiency marked growth could occur. This question, however, is clearly answered in the negative for 52 day old rabbits by Experiments 1, 2, and 3, in which all were from the same litter. Two had about one-fourth of the spleen removed at the time of transplantation and at the first examinations 80 and 52 days later both were ++, while the third, which had only one-eighth of the

[9] There are considerable variations in the size of the spleen in apparently healthy rabbits, just as in other animals, which cannot be accounted for with our present knowledge of physiology and pathology.

TABLE II.

Autotransplantation of Spleen (Complete Removal of Spleen).

Rabbit No.	Series No.	Age.	Weight.	Sex.	Date of splenectomy (complete).	Date transplanted.	First examination.	Second examination.	Final examination.	Additional data.
		days	*gm.*		*1917*	*1917*				
1	4-45	26	330	M.	Jan. 18	Jan. 18	R. 54+++	R. 145+++	R. 278+++	Jan. 18. L. adrenalectomy.
							L. 54+++	L. 145+++	L. 278+++	May. 1. Thyroidectomy.
2	4-46	26	315	"	" 18	" 18	R. 54+++	R. 139+++	R. 141+++	Jan 18. L. adrenalectomy.
3	4-47	33	575	"	" 25	" 25	" 47++	" 96+++	" 230+++	" 25. " "
							L. 47++	L. 96+++	L. 230+++	H_2SO_4. May 1. Thyroidectomy.
4	4-48	33	505	F.	" 25	" 25	R. 47++	R. 132+++	R. 134+++	Jan. 25. L. adrenalectomy.
5	4-51	40	330	"	Feb. 1	Feb. 1	" 40++	" 110++++	" 224++	Feb. 1. " "
							L. 40++	L. 110+++	L. 224++	H_2SO_4. May 1. Thyroidectomy; transplants slightly decreased.
6	4-52	40	415	M.	" 1	" 1	" 40++	" 125++	" 232+++	Feb. 1. L. adrenalectomy. Thyroids markedly enlarged at autopsy.
7	4-53	47	405	F.	Jan. 30	Jan. 30	R. 42++	R. 226++++	R. 384++++	Jan. 30. L. adrenalectomy;
							L. 42++	L. 226++++	L. 384++++	H_2SO_4. May 1. Thyroidectomy.
8	4-63	54	410	"	Feb. 6	Feb. 6	R. 35++	R. 84+++	R. 121+++	Feb. 6. L. adrenalectomy;
							L. 35++	L. 84+++	L. 121+++	KOH. May 1. Thyroidectomy.
9	4-64	54	310	"	" 6	" 6	" 35++	" 121++	" 133++	Feb. 6. L. adrenalectomy. Thyroids small.

10	4–73	61	555	F.	Feb. 13	Feb. 13	R. 58++++ L. 58++++	R. 77++++ L. 77++++	R. 119++++ L. 119++++	Feb. 13. L. adrenalectomy; KOH. May 1. Thyroidectomy.
11	4–94	61	595	"	" 13	" 13	" 58+++	" 114+++	" 115+++	Feb. 13. L. adrenalectomy. Thyroid normal.
12	4–38	66	1,275	M.	*1916* Dec. 7	*1916* Dec. 7	" 36+	" 82++	" 183++	Dec. 6. L. adrenalectomy; phosphoric acid.
13	4–37	66	875	F.	" 7	" 7	" 36+	" 56++	" 82+++	Dec. 6. L. adrenalectomy; phosphoric acid.
14	4–36	66	1,225	M.	" 7	" 7	" 36+	" 56+	" 181++	Dec. 6. L. adrenalectomy; phosphoric acid. Feb. 27, 1917. Thyroidectomy.
15	4–86	75	790	"	*1917* Feb. 27	*1917* Feb. 27	R. 44++ L. 44++	R. 63+++ L. 63+++	R. 197++++ L. 197++++	Feb. 27. L. adrenalectomy; KOH. May 1. Thyroidectomy.
16	4–26	83	1,620	"	*1916* Dec. 5	*1916* Dec. 5	" 38++	" 290+++	" 1,181+++	Dec. 5. L. adrenalectomy; phosphoric acid; double ligation of vas deferens. Apr. 5, 1917. L. gonadectomy; partial thyroidectomy.
17	4–25	83	1,450	F.	" 5	" 5	" 38+	" 84+++	" 185+++	Dec. 5. L. adrenalectomy; phosphoric acid; partial thyroidectomy.
18	4–110	84	1,050	"	*1917* May 8	*1917* May 8	R. 31++ L. 31++	R. 141++++ L. 141++++	R. 679+++ L. 679+++	Pregnant; thyroids normal.

TABLE II—*Concluded.*

Rabbit No.	Series No.	Age.	Weight.	Sex.	Date of splenectomy (complete).	Date transplanted.	First examination.	Second examination.	Final examination.	Additional data.
		days	*gm.*		*1917*	*1917*				
19	4-112	84	1,090	M.	May 8	May 8	R. 31+ L. 31+		R. 141+ L. 141+	Thyroids normal; lack of growth of transplants; large accessory spleen.
20	4-113	84	1,225	"	" 8	" 8	R. 31++ L. 31++	R. 141+++ L. 141+++	R. 475++++ L. 475++++	Example of acute enlargement of spleen transplant with pneumonia. White blood corpuscles 30,800.
21	4-94	90	1,195	"	Mar. 22	Mar. 22	R. 77+ L. 77+		R. 175++ L. 175++	Mar. 22. L. adrenalectomy.
22	4-95	97	1,200	F.	Apr. 3	Apr. 3	R. 64++ L. 64++	R. 176+++ L. 176+++	R. 1,062+++ L. 1,062+++	
23	4-97	104	1,265	M.	" 10	" 10	R. 58+++ L. 58+++		R. 169+ L. 169+	Spleen much enlarged at time of removal; evidence that transplants grew more rapidly, then decreased with recovery of animal.
24	4-103	111	1,275	"	" 17	" 17	R. 51++ L. 51++		R. 163++ L. 163++	
25	4-119	132	2,240	"	June 7	June 7			R. 111+ L. 111+	Failure to obtain growth with spleen removed.
26	4-77	167	3,025	"	Feb. 15	Feb. 15	R. 56+ L. 56+	R. 75+ L. 75+	R. 209+ L. 209+	Feb. 15. L. adrenalectomy; KOH. May 1. Thyroidectomy.

27	4-78	167	3,445	M.	Feb. 15	Feb. 15	R. 56−		R. 113−	Feb. 15. L. adrenalectomy; enlarged thyroid lobes. Failure to take probably due to error in technique.
28	4-84	173	2,575	F.	" 22	" 22	" 49+ L. 49+	R. 68+ L. 68+	" 106+ L. 106+	Feb. 22. L. adrenalectomy; KOH; thyroidectomy.
29	4-85	173	3,425	"	" 22	" 22	R. 49+ L. 49+	R. 68+	R. 202+	Feb. 22. L. adrenalectomy; KOH.
30	4-79	320	4,835	"	" 15	" 15	R. 56++ L. 56++		" 61++ L. 61++	Died; pneumonia.
31	4-80	320	4,435	"	" 15	" 15			R. 23+ L. 23+	" "
32	4-83	320	2,940	M.	" 20	" 20	R. 51+		R. 107+	Feb. 20. L. adrenalectomy; thyroidectomy. Apr. 3. Gonadectomy.
33	4-68	396	2,525	"	" 8*	" 8	" 33+ L. 33+	R. 114+ L. 114+	" 216+ L. 216+	June 22, 1916. R. adrenalectomy. Oct. 10. L. gonadectomy. Apr. 11, 1916. Thyroidectomy.
34	4-71	396	2,790	"	" 8	" 8	R. 33+ L. 33+		R. 118+ L. 118+	Feb. 12, 1916. Thyroidectomy. Nov. 3. R. adrenalectomy.
35	4-72	586+	3,150	"	" 8	" 8	R. 33+ L. 33+		R. 118+ L. 118−	Apr. 5. Gonadectomy. Oct. 25, 1915. Thyroidectomy.

* Small accessory spleen left.

TABLE III.

Autotransplantation of Spleen.

Rabbit No.	Series No.	Age.	Weight.	Sex.	Date of splenectomy.	Date transplanted.	First examination.	Second examination.	Final examination.	Additional data.
		days	*gm.*		*1917*	*1917*				
1	4-92	52	720	M.	Mar. 20. $\frac{1}{4}$ removed.	Mar. 20	R. 80++ L. 80++		R. 177+++ L. 177+++	Mar. 20. L. adrenalectomy. Thyroid normal.
2a	4-93	52	700	F.	Mar. 20. $\frac{1}{4}$ removed.	" 20	R. 52++ L. 52++		R. 177+++ L. 177++++	Mar. 20. L. adrenalectomy. Thyroid normal.
b					Apr. 17. $\frac{1}{4}$ removed.	Apr. 17	R. 52++		R. 150++	
3a	4-90	52	635	M.	Mar. 20. $\frac{1}{8}$ removed.	Mar. 20	" 80+ L. 80+			Mar. 20. L. adrenalectomy.
b					June 9. Remainder removed.				" 177++++ L. 177++++	Very little growth in first 80 days; marked after removal of spleen; compensatory.
4a	4-88	64	1,050	"	Mar. 20. $\frac{1}{8}$ removed.	Mar. 20	R. 49+ L. 49+			Mar. 20. L. adrenalectomy.
b					May 8. Remainder removed.			R. 79+++ L. 79+++	R. 177+++ L. 177+++	Thyroid slightly enlarged; very little growth in first 80 days; marked after removal of spleen.
5a	4-89	64	985	"	Mar. 20. $\frac{1}{8}$ removed.	Mar. 20	R. 49+ L. 49+			Mar. 20. L. adrenalectomy.
b					May 8. Remainder removed.			R. 79++ L. 79++	R. 177+++ L. 177+++	Thyroid vascular; slightly enlarged. Rapid growth after removing spleen.

6	4–104	80	960	M.	Apr. 17. $\frac{1}{8}$ removed.	Apr. 17	R. 53+		R. 163+	No growth with spleen intact.
7	4–105	80	1,055	"	Apr. 17. $\frac{1}{8}$ removed.	" 17	" 53+		" 196+	No growth with spleen intact.
8	4–106	80	1,030	"	Apr. 17. $\frac{1}{8}$ removed.	" 17	" 53+		" 163+	No growth with spleen intact.
9	4–114	84	1,260	F.	May 8. $\frac{1}{8}$ removed.	May 8	" 31+ L. 31+		" 141+ L. 141+	No compensatory growth of transplants.
10	4–115	84	1,075	"	May 8. $\frac{1}{8}$ removed.	" 8	R. 31+++ L. 31++		R. 141++ L. 141+	Thyroid vascular and enlarged.
11	4–107	87	1,110	"	Apr. 24. $\frac{1}{8}$ removed.	Apr. 24	R. 52+ L. 52+		R. 163+ (very small). L. 163+ (very small).	Failure to obtain growth without spleen removal.
12	4–108	87	1,010	M.	Apr. 24. $\frac{1}{8}$ removed.	" 24	R. 52+		R. 198+	Failure to obtain growth without spleen removal.
13	4–96	97	1,245	"	Apr. 3. $\frac{1}{4}$ removed.	" 3	" 64+ L. 64+		" 176+ L. 176+	No compensatory hyperplasia.
14	4–98	104	985	F.	Apr. 10. $\frac{1}{2}$ removed.	" 10	R. 58+ L. 58+		R. 169+ L. 169+	Decreased with recovery of animal. Partial removal failed to produce growth.
15	4–118	124	1,625	M.	May 17. $\frac{1}{8}$ removed.	May 17	R. 21+ L. 21+		R. 132+ L. 132+	Failure to obtain growth of transplant with spleen intact.

spleen removed, was only + at the first examination 80 days later. The remainder of the spleen in this rabbit was then removed and at 177 days the transplants were ++++, while the first two which did not have the remainder removed were only +++ at 177 days. The same reaction is seen in Experiments 4 and 5, little if any growth occurring until the spleen was removed, when a marked growth promptly took place. The effect of splenic deficiency in stimulating the growth of transplants is striking, as is shown either by comparing animals of similar ages with and without partial removal or by comparing the growth of the transplants before and after total splenectomy in the same animal. The stimulus for this increased growth must be chemical in nature and must operate through the blood stream. The compensatory hyperplasia of the stump following partial removal of the gland cannot be separated from a possible nerve influence. The method of transplantation clearly demonstrates that specific nerves are not necessary for this reaction. Some investigators have not been able to obtain compensatory hyperplasia even of the stump *in situ* following partial removal, or of the thyroid, whose functions so far as we know cannot be assumed by any other tissue, and have doubted its occurrence, while others have no difficulty in demonstrating the effect even in transplants. In the work of Halsted[10] and Hunnicut[11] on the thyroid their failure to obtain compensatory hyperplasia was probably due to their failure to induce a thyroid insufficiency, in the production of which two factors of the utmost importance are involved; *viz.*, the amount of thyroid removed and the presence of available iodine. Loeb[12] recently published his results on compensatory hypertrophy of the thyroid following partial removal in guinea pigs. He found that compensatory hypertrophy occurs, though it is necessary to remove nearly all the gland. We have found that in rats, rabbits, and guinea pigs it is necessary to

[10] Halsted, W. S., Hypertrophy of the thyroid gland. Revision of experiments made 25 years ago, *Proc. Soc. Exp. Biol. and Med.*, 1912–13, x, 111.

[11] Hunnicut, J. A., The absence of hyperplasia of the remainder of the thyroid in dogs after piecemeal removal of this gland. Auto-transplantation of the thyroid in partially thyroidectomized animals, *Am. J. Med. Sc.*, 1914, cxlviii, 207.

[12] Loeb, Leo, Studies on compensatory hypertrophy of the thyroid gland. I, *J. Med. Research*, 1919, xl, 199.

remove relatively more thyroid in order to obtain compensatory hyperplasia of the remaining stump than in cats and dogs, and in all cases available iodine must be excluded because of its inhibitory effect.[13] We believe that Loeb would have obtained more constant results had he in all instances allowed an interval of 30 days for compensatory hyperplasia to take place.

Histology of the Transplants.—This has been described elsewhere[1] and only brief mention of certain features need be made. Many of the transplants have reached 5 or 6 mm. in diameter (intracapsular measurements). They have all the general characteristics of normal spleen, both as to the number of component structures—capsule, trabeculæ, lobules, Malpighian bodies, pulp, sinuses, blood pigment—and their relation to each other. No attempt has been made to demonstrate the presence or absence of smooth muscle fibers in the capsule and trabeculæ. Apart from this the spleen is capable of complete regeneration. These studies indicate that while anatomically the spleen is very complex, biologically all the major elements are simple and endowed with uniform and marked regenerative capacity.

Permanence of Spleen Grafts.—Most of the experiments were terminated within a year. Two rabbits (Nos. 16 and 22) have been allowed to survive and at the examination on March 1, 1920 the transplants in each were found active and very vascular—1,181 and 1,062 days respectively. They are possibly slightly smaller than at the second examination 290 and 176 days after transplantation. Both rabbits were young at the time of transplantation and in both complete splenectomy was performed, thus insuring growth of the transplants. Both rabbits are still strong and active. One can conclude, therefore, that spleen autografts made under conditions which insure good initial growth are permanent. There appears, however, to be a slight involution or atrophy with age even in splenectomized rabbits, and, as already pointed out, transplants made in old rabbits without splenectomy may in time (several months) undergo complete atrophy. Our experiments with autotransplan-

[13] Marine, D., and Lenhart, C. H., Colloid glands (goiters): their etiology and physiological significance, *Bull. Johns Hopkins Hosp.*, 1909, xx, 131.

tation of the spleen and also of the thyroid show that at least an anatomical deficiency is not necessary in order that transplants may take and remain active for several months. Growth of these grafts, however, usually does not occur unless there is a physiological insufficiency which may exist independent of the amount of functionally active organ. Loeb and Hesselberg[14] have also shown that the taking of transplants is independent of a physiological insufficiency. Halsted,[15] working with the parathyroid, concluded that it was necessary to induce a physiological insufficiency in order to obtain successful transplants.

Reaction of the Grafts in Acute Infections.—No experiments have been made relative to this point. In a few instances in which the rabbits died of pneumonia, the transplants were markedly congested, and in one rabbit (Experiment 19) which died of pneumonia, the transplants at autopsy were soft, engorged with blood, and microscopic examination showed increase in pulp cells. In the ordinary sporadic cases of pneumonia in rabbits the reaction of the spleen is so variable and even in healthy rabbits there are such variations that it would be necessary to carry out a series of experimentally controlled infections to obtain definite data.

SUMMARY.

No instance of survival of spleen homeografts beyond the usual taking and persistence for 1 or 2 weeks common to most homeografts has been observed, although the possible advantages of consanguinity, age, and splenectomy were fully utilized. This is in sharp contrast to thyroid, sex gland, and adrenal cortex homeografts, with which one may expect 10 per cent to survive the 30 day period. It suggests that spleen is a stronger antigen and excites a greater degree of immunity more quickly. With autografts survival and growth are the rule, and failures are due to technical errors. Age is an important factor in the growth of autografts. The younger the rabbit the

[14] Loeb, Leo, and Hesselberg, C., Studies on compensatory hypertrophy of the thyroid gland. II, *J. Med. Research*, 1919, xl, 265.

[15] Halsted, W. S., Auto- and isotransplantation, in dogs, of the parathyroid glandules, *J. Exp. Med.*, 1909, xi, 175.

more growth is aided. This beneficial effect decreases gradually and becomes negligible after sexual maturity. Removal of the spleen is a powerful stimulus to the growth of transplants. The effect varies inversely with the age and usually is negligible after sexual maturity. The influence of age and splenectomy suggests that the spleen is most important in early life and after sexual maturity is either unimportant or its functions may readily be assumed by other tissues (hematopoietic). Anatomically the spleen is a highly complex structure, but biologically all the major elements of the spleen are simple as indicated by the uniform and marked regenerative capacity. There is a tendency for grafts to involute or atrophy with age, and grafts made in old rabbits without removal of the spleen may undergo complete atrophy. Grafts made in young rabbits, accompanied by splenectomy, have been observed for more than 3 years and may be said to be permanent. There is some evidence that subcutaneous autografts react to infections in the same way as the intact spleen.

A METHOD FOR FIXING FILMS OF HUMAN BLOOD CELLS DURING THE AMEBOID MOVEMENT OF LEUCOCYTES AND THROMBOCYTES.

By M. A. VAN HERWERDEN, M.D.

(*From the Histological and Embryological Laboratory of the University of Utrecht, Utrecht, Holland.*)

PLATE 1.

(Received for publication, January 5, 1920.)

In human blood films fixed in the usual manner, the ameboid movement of leucocytes and thrombocytes is lost. I have therefore endeavored to obtain a simple method to retain this movement in the fixed specimen. Of course, this is possible only by very quick fixation at body temperature, while the cells are in an environment which does not hinder normal motility. The following method gives excellent results (Figs. 1 and 2).

A watch-glass, covered by a second watch-glass, on the inside of which is laid a filter paper moistened with water, is placed in an incubator at a temperature of 38°C. On a well cleaned cover-slip is placed a drop of Deetjen's solution,[1] previously heated to body temperature, to which is added a very small drop of blood from the finger. This cover-slip is placed in the space between the watch-glasses which is kept moist by the wet filter paper. After about 20 minutes in the incubator the covering watch-glass is quickly replaced by another one, on the inside of which a filter paper is placed moistened with a solution of 40 per cent formaldehyde. In this manner the leucocytes and thrombocytes which have continued their ameboid movement, while in the damp space, are quickly fixed at body temperature. After about half an hour the cover-slip is taken out of the incubator and the mixture of Deetjen's solution and blood is carefully run off so that part of the red blood corpuscles, leucocytes, and thrombocytes are left adhering to the cover-slip. The film can now

[1] 0.75 per cent sodium chloride, 0.5 per cent manganese sulfate, 0.01 per cent sodium bicarbonate.

be stained and treated further in the usual way. The best results are obtained if the film is stained while still wet.

In this way well fixed films are obtained. For the thrombocytes, Deetjen's solution is preferable to Ringer's solution, but often good results are also obtained with the latter. The finest ameboid projections of the multinuclear leucocytes as well as those of the mononuclear leucocytes are visible. There is sometimes a radiation of protoplasm around the nucleus of the lymphocytes; the other leucocytes are very irregular in shape. Moreover, the change of shape of the nuclei during the ameboid movement of the cell, seldom visible in the living specimen, can be studied in this way.

Comparison of the size of ameboid leucocytes with that of equally well fixed red blood corpuscles makes it evident that the impression ordinarily obtained of the size of these bodies is most incomplete. This is also true of the thrombocytes. In films made by the method described, the latter generally are of various sizes and shapes. Thrombocytes are found which are as large as red blood corpuscles; others again are much smaller, but also with ameboid projections. The nucleus is scarlet if stained according to Romanowsky's method; it is dark or light blue after being stained in hematoxylin for 24 hours; but it is most distinct if stained with Heidenhain's iron-hematoxylin. By the last method all grades of transition from small pycnotic to large pale nuclei can be observed. In some of the thrombocytes small basophil granules are given off by the nucleus, or the latter is entirely split up into granules. Among the numerous thrombocytes examined I have never been able to distinguish a mitotic division; in some films a constriction and doubling of the nucleus have been seen. In the latter case, as shown in Fig. 1, probably cell division was about to take place.

In films prepared in this way, one can study the position of the mitochondria in the ameboid leucocytes, especially if after being fixed with formaldehyde they have been left for a few days in a solution of 3 per cent potassium bichromate and have then been stained in Heidenhain's iron-hematoxylin (method of Regaud). Sometimes mitochondria and chondriocontes continue into the cell processes.

The method described above demands, beyond careful treatment, so little technical practice that it can very well be carried out by students during practical work.

It may also be interesting from the clinical point of view to observe leucocytes and thrombocytes in their natural shapes, and perhaps to examine the behavior of blood parasites by this method. The method also permits a study of the influence of different substances (poisons, etc.) upon ameboid movement. For instance, it has been noted that an isotonic solution of potassium chloride and sodium chloride, if used instead of Deetjen's solution, allows the ameboid movement to continue (treatment for 1 hour). The same result is obtained with 0.0001 N phenylurethane (in Deetjen's solution), while the same narcotic in a concentration of 0.001 N inhibits motility in less than 1 hour. These are only a few examples of the observations made.

This method has also been used to study the influence of radium radiation on the motility of leucocytes. On the cover-slip that is to carry the drop of Deetjen's solution and the drop of blood is placed a ring of paraffin about 2 mm. in height to support the radium capsule. A control specimen, prepared in like manner, is covered by a second cover-slip, instead of by the radium capsule. The rest of the procedure has been outlined above. The radium capsule used for the experiment contained 3.1 mg. of radium bromide under a mica cover. The blood mixture irradiated with this for 8 hours at body temperature contained several leucocytes which had kept up their ameboid movement. The same intensity of irradiation kills the eggs of *Daphnia pulex* in a much shorter time (generally a few minutes), as demonstrated in a former paper.[2]

In conclusion it may be mentioned that by this method it might be possible to obtain a better view than has hitherto been possible of the morphological changes of thrombocytes during coagulation of the blood.

EXPLANATION OF PLATE 1.

Fig. 1. Cells of human blood fixed during ameboid movement. The film has been stained with Heidenhain's iron-hematoxylin. *l.*, leucocyte; *thr.*, thrombocyte; *r.bl.*, red blood corpuscle. Magnification × 1,000.

Fig. 2. Blood film stained with iron-hematoxylin. At the left one multinuclear leucocyte is shown. The other ameboid bodies are thrombocytes of different sizes.

---

[2] Van Herwerden, M. A., *Proc. Koninklÿke Akad. Wetenschappen*, 1918, xxi, 540; *Genetica*, 1919, i, 305.

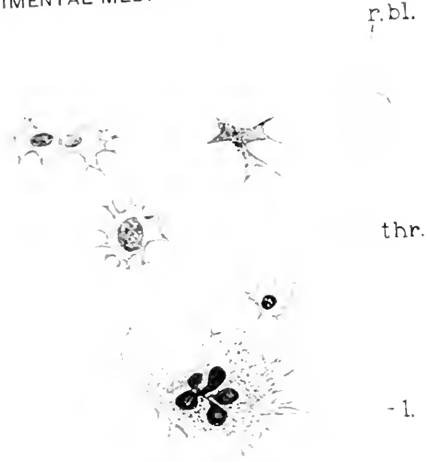


FIG. 1.

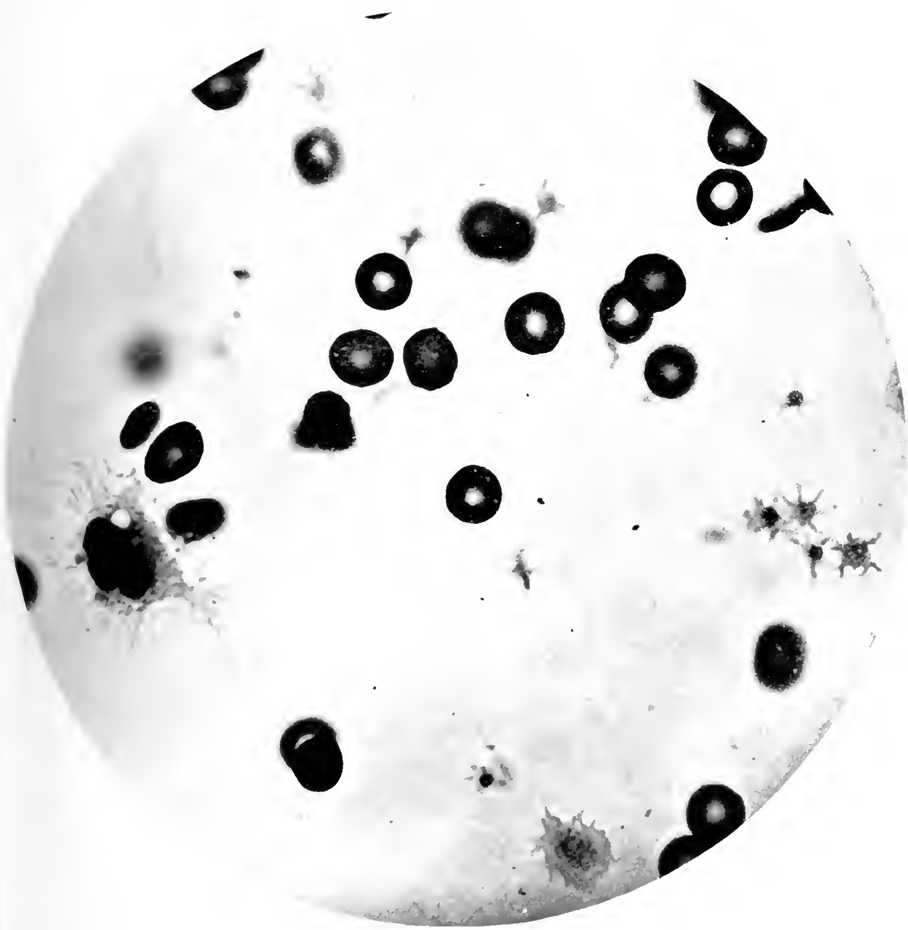


FIG. 2.

(Van Herwerden: Fixing of human blood cells.)

SEGMENTING TERTIAN MALARIAL PARASITES ON RED CORPUSCLES SHOWING LITTLE OR NO LOSS OF HEMOGLOBIN SUBSTANCE. EVIDENCE OF MIGRATION.*

By MARY R. LAWSON, M.D.

(*From the Laboratory of Dr. Mary R. Lawson, New London.*)

PLATES 2 AND 3.

(Received for publication, April 12, 1920.)

Migration of Malarial Parasites.

The malarial parasite has been described as attaching itself when young to a red corpuscle and remaining there during the entire period of its life cycle, the destruction of the infected corpuscle corresponding to the segmentation of the parasite. But this is not so. Each parasite destroys several red corpuscles, migrating to a fresh corpuscle as soon as one is destroyed. I have seen migrating parasites in eight different stages of development in tertian infections, the oldest form being the presegmenting parasite (Figs. 1 to 5).

Segmenting Parasites Attached to Red Corpuscles Whose Hemoglobin Is Intact or Nearly So (Figs. 6 to 49).

Thayer,[1] discussing tertian parasites, states: "In certain instances, however, sporulating forms may be found within corpuscles which are no larger than the normal red cell, and which are but little decolorized." He writes that Bastianelli and Bignami[2] are inclined to believe that such bodies are more common in cases of anticipating tertian fever. But Thayer states that his observations have not been sufficient to justify

* Aided by a grant from The Rockefeller Institute for Medical Research.

[1] Thayer, W. S., Lectures on the malarial fevers, New York, 1897, 55.

[2] Bastianelli and Bignami, quoted by Thayer,[1] p 55.

the formation of a definite opinion concerning this point. He makes no attempt to explain the occurrence; but he has pictured two segmenting tertian bodies on normal appearing red cells, designating them as instances of "precocious segmentation."[3]

It seems to me that the presence of segmenting parasites on red corpuscles with hemoglobin intact or nearly so is evidence that the parasites have recently attached themselves and have not had sufficient time to cause any appreciable damage to the infected cells and the pigment in connection with these segmenting parasites is additional evidence of their previous attachments to red corpuscles.

Infected Red Corpuscles of Tertian Infections.

One of the most striking features of tertian infections is the rapidity with which the parasites enlarge and decolorize the infected cells. Even the very young parasites do this soon after their attachment, and segmenting parasites are most frequently described as attached to decolorized cells. Marchiafava and Bignami[4] write: "one of the most characteristic properties of the tertian parasite is the rapidity with which the infected red cell becomes decolorized and swollen." The infected corpuscles in the accompanying plates show such a slight amount of alteration due to parasitic action that it is obvious that they could not have been occupied by the attached parasites for the entire period of their development.

While all observers have noted that the infected corpuscle is enlarged as a result of parasitic action, there are also instances in which it is enlarged as a result of the large size of the attached segmenting body. For instance, the segmenting bodies illustrated in Figs. 22 to 25 are larger than normal corpuscles at the same magnification, and they are too large to occupy corpuscles the size of those pictured in Figs. 7 to 10 without enlarging the corpuscles. The red corpuscles being more elastic than the parasites are readily enlarged when pressure is exerted. This mechanical enlargement of a corpuscle means a thinning of its substance, consequently it is usually paler than one

[3] Thayer,[1] Plate 1, Figs. 16 and 17.

[4] Marchiafava, E., and Bignami, A., Malaria, in Stedman, T. L., Twentieth century practice, New York, 1900, xix, 63.

of normal size. The increased size of many of the infected cells shown in the accompanying plates is the result of the large size of the attached parasites rather than of parasitic action.

Segmenting Bodies of Tertian Infections.

The tertian segmenting bodies are larger than those of the quartan or æstivo-autumnal infections. Within certain limits, from about 14 to 26, they show no fixed number of segments; occasionally I have seen fewer than 14, rarely more than 26. When more than 26 are seen it means, usually, that two segmenting bodies are attached to one corpuscle. It is interesting to note that the small segmenting body does not necessarily mean that the parasite has fewer segments. The pigment in connection with these bodies is usually collected in a mass, but it may be scattered among the spores. The young parasites resulting from the segmentation of these bodies attached to healthy appearing corpuscles frequently attach themselves independently and remain until the corpuscle is destroyed.

SUMMARY.

1. Malarial parasites destroy more than one red corpuscle, migrating to another as soon as one is destroyed.

2. Pigmented segmenting parasites attached to red corpuscles whose hemoglobin is intact or nearly so are *prima facie* evidence of migration.

3. Infected corpuscles in tertian infections may be enlarged as a result of the large size of the attached parasite as well as by parasitic action.

4. The young parasites resulting from the segmenting bodies attached to healthy red corpuscles usually attach themselves independently to the infected corpuscle and remain there until the corpuscle is destroyed.

EXPLANATION OF PLATES.

Plate 2.

TERTIAN PARASITES.

Magnification × 1,807.

Figs. 1 to 5. Presegmenting parasites free in the blood serum. These are parasites in the last free stage of migration.

Fig. 6. A presegmenting parasite attached to a red corpuscle which is enlarged but with hemoglobin nearly intact.

Figs. 7 to 11. Segmenting parasites attached to red corpuscles which are practically normal both in size and hemoglobin content.

Figs. 12 to 24. Segmenting parasites attached to red corpuscles whose hemoglobin is practically intact; the cells are enlarged slightly owing, in Figs. 22 to 24, to the large size of the attached parasites.

Fig. 25. A presegmenting parasite attached to a red corpuscle which is normal in hemoglobin content but enlarged by the large size of the parasite.

Plate 3.

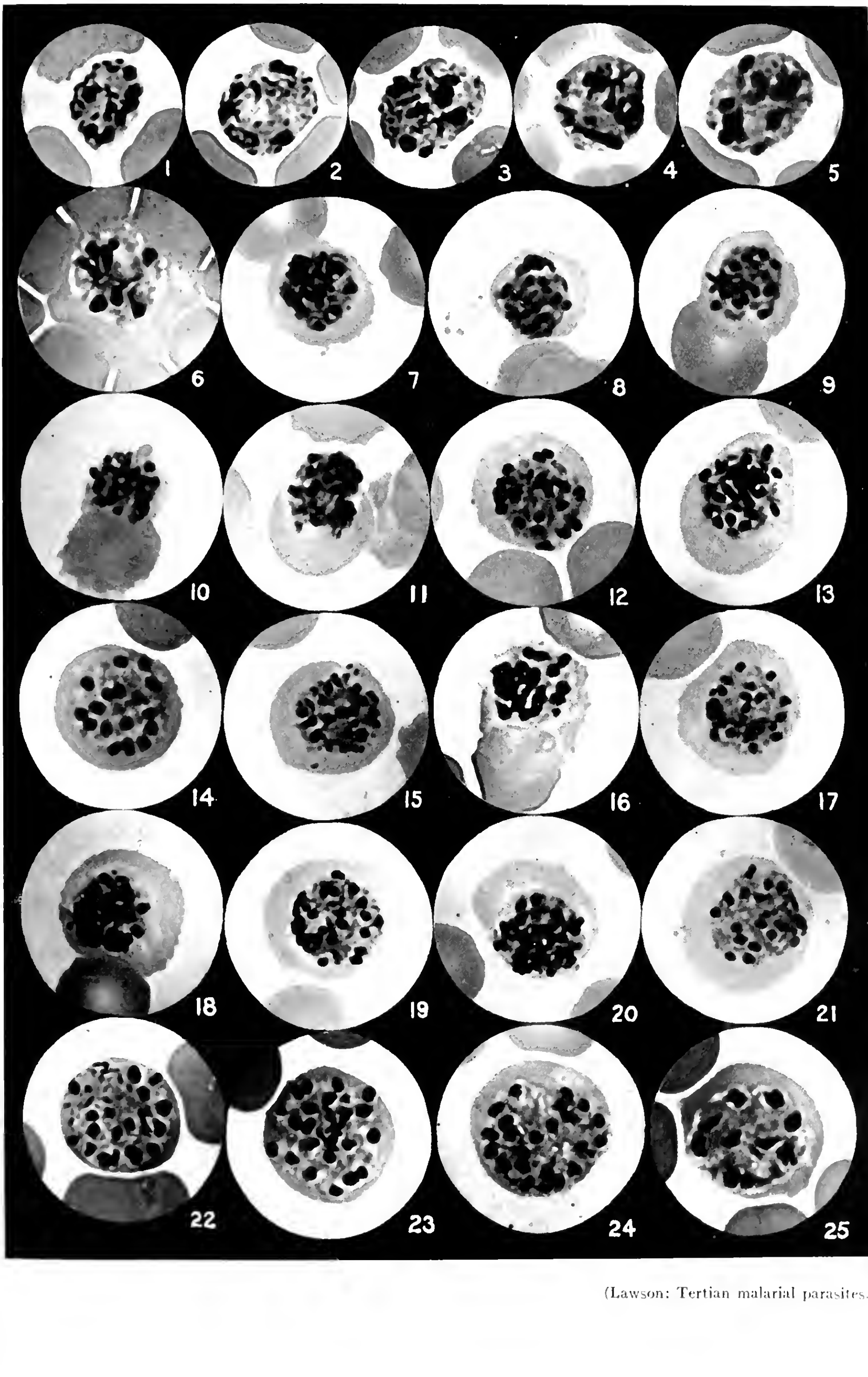
TERTIAN PARASITES.

Magnification × 1,807.

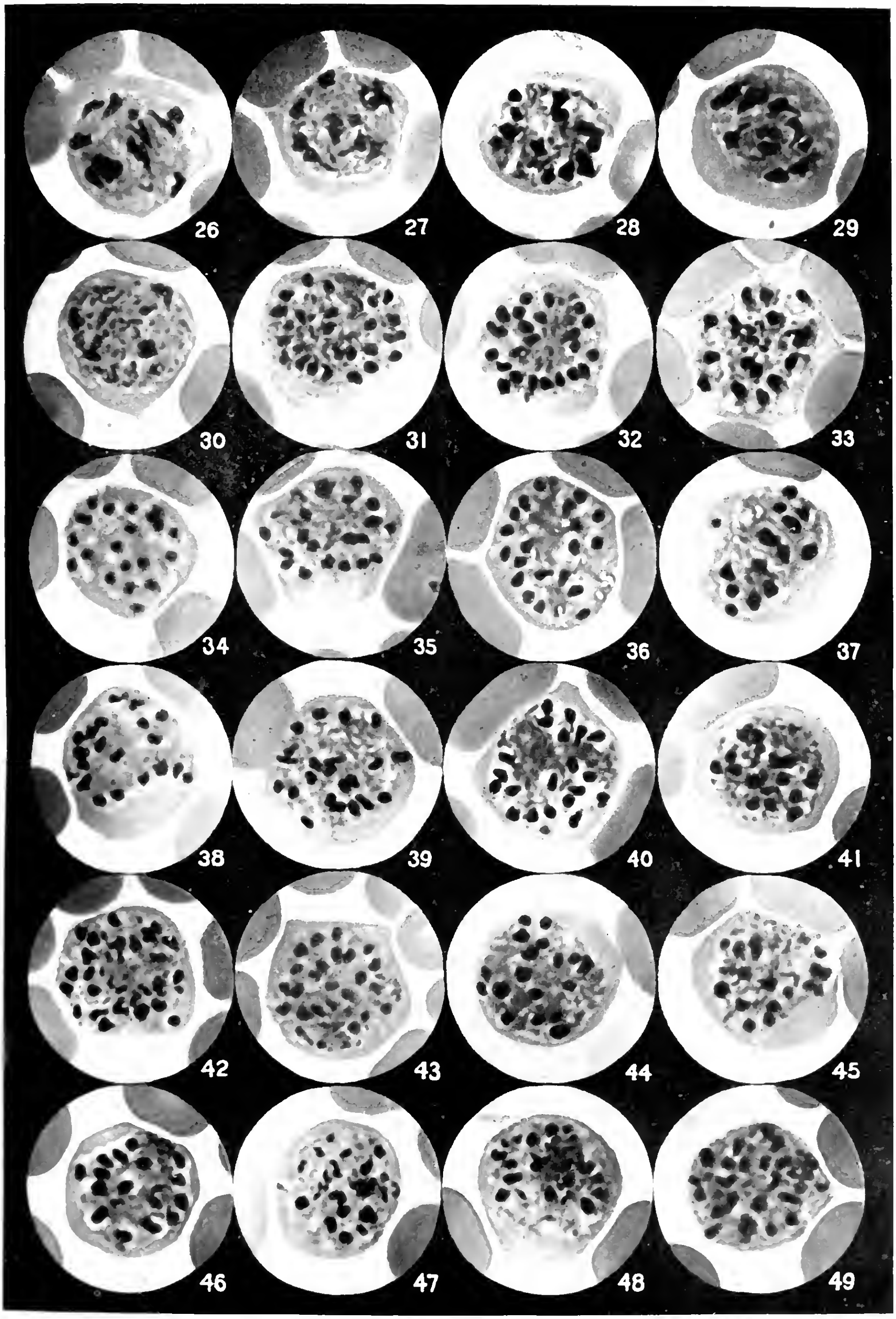
Figs. 26 to 30. Presegmenting parasites attached to normal red corpuscles which are enlarged by the large size of the attached parasites.

Figs. 31 to 33. Segmenting parasites attached to red corpuscles which show a slight alteration due to parasitic action. Note the fine stippling of the cells (Shüffner's granulation).

Figs. 34 to 49. Segmenting parasites attached to fairly healthy red corpuscles. Note the large number of segments of some of the segmenting bodies.



(Lawson: Tertian malarial parasites.)



(Lawson: Tertian malarial parasites.)

BLACKHEAD IN CHICKENS AND ITS EXPERIMENTAL PRODUCTION BY FEEDING EMBRYONATED EGGS OF HETERAKIS PAPILLOSA.

By THEOBALD SMITH, M.D., and H. W. GRAYBILL, D.V.M.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

(Received for publication, May 19, 1920.)

The occurrence of blackhead or typhlitis in chickens has been noted by several observers. To the meager literature reported by Smith[1] in 1915 the following may be added.

In 1907 Theobald[2] reported the occurrence of the disease in England. In 1908 Milks[3] observed it in four widely separated localities in Louisiana. It was not seen in birds more than 6 weeks old. The course of the disease was rapid and the mortality 30 to 50 per cent. Higgins[4] found in one chicken ulceration of one cecum which he describes as typical of enterohepatitis or blackhead in turkeys. Tyzzer[5] observed the disease in two chickens. In a chick 4 weeks old there was found a slight invasion of the cecum and extensive involvement of the liver. A slight involvement of one cecum was later found in a hen 2 years old.

During the past 3 years a few cases of disease in chickens associated with *Amœba meleagridis* were brought to the laboratory. In the brief notes given below Nos. 18 and 19 were from one flock, and Nos. 91 and 92 from another. These flocks were over 50 miles apart.

Chicken 18, White Leghorn, brought alive Apr. 18, 1917. Said to have been hatched in Feb. At the autopsy there was found an intussusception of the small intestine of which about 7 cm. were involved. This portion was in a hemor-

[1] Smith, T., *J. Med. Research*, 1915, xxxiii, 243.

[2] Theobald, F. V., *2nd Nat. Poultry Conference, Official Rep.*, Reading, 1907, 181.

[3] Milks, H. J., *Louisiana Agric. Exp. Station, Bull. 108*, 1908, 1.

[4] Higgins, C. H., *Dom. Canada, Dept. Agric., Health of Animals Branch, Bull. 17*, 1915. 1.

[5] Tyzzer, E. E., *J. Med. Research*, 1919, xl, 1.

rhagic condition. Each cecum contained a consistent fecal mould coated with a thin but tough, partly white, partly grayish exudate. The walls of the ceca were thickened, opaque. The liver contained quite small, scattering, indistinctly outlined, yellowish foci. Sections of the ceca showed complete loss of the mucosa which was replaced by a dense layer of fibroblastic tissue. Below this layer in the muscular coat and extending to the serous covering, there were numerous vacuoles of various sizes in the tissue which contained each from one to a dozen or more roundish bodies about 6μ in diameter. Each contained a nucleus with a minute deeply stained caryosome in state of binary division in some of the organisms. In the liver sections the foci seen at autopsy were about one-third the diameter of a lobule. They consisted of necrotic liver cells, parasites like those of the cecal walls, chiefly within giant cells, and many lymphoid elements.

Chicken 19, White Leghorn, received at the same time and from the same flock. The ceca were affected as in No. 18, but the liver was free from foci. Sections of the ceca showed the same kind of changes as in No. 18, but portions of the mucosa were still present. Parasites were rare and seen only in some of the many vacuoles scattered through the superficial fibroid substitute for the destroyed parts of the mucosa. These vacuoles probably lodged parasites now destroyed.

Chicken 91, Rhode Island Red, had been killed before reaching the laboratory. Female, weighing 500 gm. Both ceca contain consistent cores of fecal material which are not adherent to the wall. The underlying mucosa is smooth and free from necrotic areas. The liver is enlarged and covered with opaque whitish foci 6 to 7 mm. in diameter. Some contain minute yellowish specks in the whitish ground. In teased preparations from these foci numerous homogeneous, roundish bodies, about 12μ in diameter, are seen. These are not distinguishable from *Amœba meleagridis* found in diseased turkeys. In sections of the ceca, the thickening of the wall is due to a marked increase of plasma-like cells in the intertubular tissue. Small groups of *Amœba meleagridis* are scattered through the mucosa and one large group is located in the muscular portion of the intestinal wall. Coccidia are scattered through the core. Sections of the liver contained large numbers of *Amœba meleagridis*. This organ was involved as extensively and intensively as in fatal cases of blackhead in turkeys, while the lesions of the ceca were slight.

Chicken 92, Rhode Island Red, was received dead from the same flock. In this bird coccidiosis predominated and the only traces of blackhead found were two microscopic foci in the liver tissue crowded with *Amœba meleagridis*.

From the preceding quotations and studies it is obvious that fatal blackhead is relatively rare in chickens. The data indicate that it may be a quite common disease which passes unnoticed because partial or total recovery is the rule. The injury to the mucous membrane as shown in Cases 18 and 19 may prevent the recovered bird from reaching normal full development. The lesions and the asso-

ciated parasite appear to be identical with those observed in turkeys. The disease shows, however, greater variations from case to case. In some the liver is the chief seat and the ceca almost intact. In others the reverse is true.

The Experimental Production of Blackhead.

Following the successful attempts to produce blackhead in turkeys by feeding embryonated eggs of *Heterakis papillosa*,[6] similar experiments were made on chickens.

Experiment 1.—The chickens were hatched in an incubator. The eggs before incubation had been washed to remove any adhering dirt, placed in 0.5 per cent bichloride of mercury for 30 seconds, washed again, and dried. During the experiment the chicks were kept in brooders within isolation units, protected against vermin and infectious material likely to reach them if kept in the open. The only source of infection to which they were exposed was the grain and sour milk fed regularly with cooked food.

The worms were obtained from the ceca of two adult hens penned with old turkeys in an outdoor enclosure since the fall of 1919. To liberate the ova, the females were cut up in a Petri dish containing a shallow layer of physiological salt solution. In this they were incubated at room temperature. The feeding was done Mar. 1, when the cultures were 14 days and the chicks 32 days old. Four White Leghorns, four Plymouth Rocks, and four Rhode Island Reds were fed the ova mixed with the food in cages. After the feeding their feet were thoroughly washed and they were placed in a brooder in an isolation unit. From the same hatch nine chickens were held as controls in another unit. The chickens were killed and autopsied at certain intervals after the feeding. In case the ceca were diseased, one was opened and examined as to contents and condition of the mucosa and the other simply incised and then placed in Zenker's fluid for future study.

Chickens killed respectively 1, 2, 3, and 7 days after feeding were found normal, except for weakness of the legs, due presumably to confinement. Sections of one cecum and of liver tissue showed normal conditions. The contents of the ceca consisted of bacteria, a few food remnants, and some *Heterakis* larvæ. The walls were free from infiltrations and contained only the usual number of lymphoid cell groups. In the livers a few small compact groups of cells resembling lymphocytes were present.

In No. 279, killed 10 days after feeding, both ceca were distended and contained a firm reddish core replacing the usual soft contents. The walls of the non-villous portion were quite uniformly thickened to about 1 mm. Larval worms

[6] Graybill, H. W., and Smith, T., *J. Exp. Med.*, 1920, xxxi, 647.

(*Heterakis papillosa*) were found in the lumen. The liver was of normal appearance. Transections of one cecum fixed without disturbing the core showed a number of changes. The core, replacing the normal fecal mass, consisted almost wholly of red blood cells, only a few of which had retained the hemoglobin. There were also some necrotic tissue cells in the mass. Throughout the clot were small colonies of bacteria and a few larval forms of *Heterakis*. The mucosa was greatly altered. In several places it was completely destroyed. Elsewhere, the tubules were scarce. They were distorted and oblique to the surface. The mucosa and submucosa contained large groups of lymphocytes and strands of the same were lying in the muscular tissue. Parasites resembling *Amœba meleagridis* were abundant in the mucosa and submucosa. Many were within giant cells. The muscular coats were free. Sections of a small nematode were found in the intertubular tissue, measuring about 0.05 mm. in diameter. In the liver there were fairly numerous dense collections of lymphocytes up to 0.15 mm. diameter. Parasites could not be detected in these foci.

No. 280, killed 11 days after feeding, presented the same lesions. Neither this nor the preceding bird had shown evidences of disease. The core in the cecum was like that of No. 279. The wall presented certain differences, however. The epithelium and the tubules were normal and intact. The submucosa was markedly edematous and the lymphoid tissue somewhat increased. The blackhead parasite had permeated quite generally and distended the intertubular tissue of the mucosa and had penetrated into the submucosa in large numbers. Worms were seen in the mucosa and the lumen. The cell foci as described under No. 279 were present in the liver of this case.

Chicken 281 was killed 14 days after feeding ova. It had not shown signs of illness. The contents of one cecum were normal and contained larval worms. The wall was possibly slightly thickened and the mucosa sprinkled with minute hemorrhages. Sections of the other cecum showed conditions differing both from the preceding and the following case. The contents were normal. A small portion of the wall was normal, the rest thickened. The tubules and surface epithelium were intact. One larval worm was found partly embedded in a tubule. The increased thickness of the wall was due chiefly to a great increase in lymphoid cell groups in the submucosa. In the mucosa there was a slight diffuse infiltration of plasma cells. The muscular coat was not involved. *Amœba meleagridis* occurred in groups of two to six or more individuals in tissue spaces. The parasites were relatively scarce as compared with No. 279. The liver contained minute collections of lymphoid cells, from one to two in a field of the 16 mm. objective.

Chicken 282 was killed 15 days after feeding. It had not appeared quite normal. Both ceca were distended and firm to the touch, owing to the presence of hemorrhagic cores replacing normal feces. The walls were 2 to 4 mm. thick. Larval worms were present. The core was made up of a mass of red corpuscles embedded in a homogeneous feebly stained matrix. In one spot it was incorporated with the wall and here the mucosa was destroyed. The rest of the mucosa was covered with epithelium but the tubules were partly destroyed, partly dis-

torted. The intertubular tissue was infiltrated with lymphoid cells and amebæ. The submucosa was markedly edematous. At irregular intervals it contained large dense collections of cells enclosing amebæ. In a few places the muscular coat was infiltrated with lymphocytes.

Chicken 285, killed 18 days after feeding, had not shown symptoms of illness. It was normal as to ceca. There were a few whitish foci 1 to 2 mm. in diameter in the liver. Transverse sections of fixed and hardened tissue from four different levels of one cecum showed the same changes. There were a cellular infiltration and increase of lymphoid tissue in the mucosa and a few tubules were markedly distended. Just below the muscularis there was a dense zone or layer of cells, probably lymphocytes, and roundish masses of the same, suggesting newly formed follicles. There was no core or mass of exudate in the lumen and there were no parasites in the tissues. In the liver sections, besides the minute collections of lymphoid cells, there are a few larger groups of the same type of cells enclosing necrotic liver tissue. *Heterakis* larvæ were in the ceca.

Chicken 286, killed 23 days after feeding, had not shown symptoms. The contents of the ceca were normal and larval worms were present. There was evidence of increased numbers of cells, resembling plasma cells in the mucosa. Lymphoid follicles were more numerous than normally in the submucosa, but the diffuse infiltration of lymphocytes was lacking.

Chicken 287 was killed after 28 days. It had not shown symptoms. One cecum contained some *Heterakis* larvæ, but contents were normal. Sections of the other cecum showed slight irregularity and occasional loss of tubules, their places being filled with cells of lymphocyte type. In the intertubular tissue there was an increase of plasma-like cells. The submucosa contained more than the normal number of lymph follicles. There was one group of eighteen follicles. In the liver certain whitish foci, 1 mm. in diameter, seen at the autopsy, were found to be made up entirely of cells of lymphocyte type. A central mass about 0.5 mm. diameter was enclosed in a ring of follicles. Many small foci of similar cells, 0.1 to 0.15 mm. diameter, frequently filling out vessels were also present.

Chicken 288 was killed after 29 days. *Heterakis* larvæ in ceca. Although the animal had shown no distinct signs of disease and the organs appeared normal with the exception of a few whitish areas on the liver 1 to 2 mm. in diameter, the walls of the ceca were not normal. The mucosa had the usual number of tubules with the exception of about one-tenth of the circumference, in which the tubules were replaced with lymphoid cells. There was a general increase of cells of plasma type between the tubules and the number of follicles in the submucosa was increased. In a section of the liver there were two foci, one consisting of a plug of cells in a vessel, the other a group of cells simulating four lymph follicles surrounding a small vessel also plugged with lymphoid cells.

Putting the data of this experiment together, we observe that as a result of hemorrhages the contents of the ceca appeared as firm cores

on the 10th to the 15th day inclusive, but not before the 10th, nor after the 15th day. Protozoan parasites were in the walls of the ceca within the same period. A larger number of cases examined at shorter intervals will define these limits more accurately.

Of the controls, which had remained well with the exception of some lameness due to confinement, two were killed 17 days after the other lot had been fed, and one 30 days after. None showed signs of lesions and worms were not found.

Experiment 2.—In this experiment, which is similar to Experiment 1, certain controls were introduced. The chickens were obtained from a poultry farm just after they had been hatched and placed in a brooder in an isolation unit. They were fed as were those in Experiment 1. The worms used were obtained from the same source as those used in Experiment 1. The cultures were prepared in the same way as heretofore. Three groups were included, each consisting of four White Leghorn chickens. Group I was fed with cultures made by cutting up adult female *Heterakis* to permit eggs to escape. Group II received cultures prepared by cutting up only males. Group III was fed with sediment from the washed contents of the ceca after the worms had been removed and the contents run through a wire screen of No. 300 mesh which did not permit the few free ova to pass through. At the time of feeding, the cultures were 20 days and the chickens 18 days old. All of the chickens in Group I were diseased, as the following notes show.

On the 9th day after feeding No. 290 was not quite normal. Its head was drawn back and its wings drooped slightly. It was chloroformed next day. The body was in good condition and only the ceca were affected. They were distended to about 1 cm. in diameter, firm to the touch, and hemorrhagic at the distal end. One, cut open, contained a spongy pink and reddish core slightly adherent to the hemorrhagic mucosa. The liver showed some whitish specks. Transverse sections from the other cecum showed the presence of a core made up of a meshwork of a homogeneous substance containing some red corpuscles. It was attached to the greater part of the wall and the mucosa was here destroyed. The remaining mucosa was low, with continuous epithelium and distorted tubules. The submucosa was broadened by infiltration with large numbers of *Amœba meleagridis* and lymphoid cells. The muscular layers were also infiltrated with cells, even into the mesentery. Larvæ were present in the mucosa and lumen. In the liver sections only minute groups of lymphocytes were present.

Chicken 295 was not quite normal on the 11th day, when it was chloroformed. The gross appearance of the ceca and the condition of the wall and core of one cecum as shown in sections were so like those of No. 290 that a detailed statement is omitted. In the liver a necrotic focus with a few *Amœba meleagridis* in it deserves mention.

Chicken 296 was killed on the 12th day. It had not shown any distinct signs of illness. Both ceca were distended and firm. One was opened and a long, cylindrical, non-adherent core, gray and brown in color, removed. The wall was about 2 mm. thick. In stained sections, the core was found composed chiefly of exudate cells (lymphocytes). It was attached at one spot to the wall and here the mucosa had been destroyed. Elsewhere the low mucosa was covered with epithelium. Normal tubules were scarce. The rest were much dilated and filled with cell debris. The mucosa, submucosa, and muscular layers were indistinguishable, owing to a general invasion of *Amœba meleagridis* and a general infiltration of cellular elements, lymphocytes, and plasma cells. There were also in the muscular coat masses of cells simulating lymph follicles. In the liver there were the usual minute collections of lymphocytes and some necrotic foci, but parasites were not seen in them.

The 4th and last chicken of Group I, No. 298, was killed 13 days after feeding. It had been clinically normal. Both the macroscopic and microscopic pictures were so like those of No. 296 that no detailed description is given. The invasion of *Amœba meleagridis* was slight below the submucosa, but the infiltration of the musculature with large masses of lymphocytes, even into the mesentery, was striking.

In three of the foregoing cases larval worms were found in the contents of one cecum when examined fresh. In the fourth they were demonstrated in transections of the core of the other cecum.

The four chickens of Group II remained normal until they were killed on the 10th, 11th, 13th, and 13th day, respectively. No lesions were found and worms could not be detected. The same was true for the chickens of Group III, which were killed on the 10th, 11th, 12th, and 13th day, respectively.

DISCUSSION.

The foregoing experiments demonstrate that a disease of the ceca very closely resembling that of the enterohepatitis or blackhead of turkeys can be produced in chickens still in the brooder by feeding an overdose of embryonated eggs of *Heterakis papillosa.* The lesions appeared on or about the 9th day after feeding. Chickens killed after 15 days showed, in spite of an intact mucosa, signs of a past inflammation in the presence of large numbers of cells of the lymphocyte type in mucosa and submucosa and in the increased number of roundish accumulations of similar cells resembling lymph follicles.

The active disease manifests itself in a pouring out of blood into the lumina of the ceca, which coagulates into a firm spongy core. Later the red corpuscles disappear and lymphocytes emigrate from the

injured mucosa to form the outer zone of the core. This is attached in part to the wall and here the mucosa is necrotic. The walls of the ceca are thickened up to 4 mm. in diameter. The thickening is due to edema, infiltration of lymphocytes and of *Amœba meleagridis* in large numbers. The mucosa is more or less injured in addition to the necrosis where the core is adherent. The tubules are distorted, dilated, and in part missing. Some are filled with cell debris. In all cases there is a diffuse infiltration of plasma-like cells between the tubules. Occasional hemorrhages into the substance of the mucosa are present.

The liver, besides containing in all cases microscopic focal collections of lymphocytes, shows rather infrequently barely visible yellowish specks which consist either of necrotic foci in which giant cells and cells of endothelial type are replacing the liver cells or else of collections of lymphocytes in the form of roundish follicles or within vessels. *Amœba meleagridis* was seen in one case in a necrotic focus. On the whole, the changes in the liver so formidable in turkeys are insignificant.

The protozoan parasites presented the same morphological characters shown in the turkey's tissues. They occupied the tissue spaces in mucosa and submucosa and more rarely in the muscular coat. As a rule, the bodies of the parasite appeared as if in a state of disintegration except in No. 281, in which they had a homogeneous cytoplasm. In some cases most of them were within phagocytic cells. In one case (No. 290) the protozoa set free from teased portions of the mucosa showed, without the use of a warm stage, finger-like pseudopodia in continual change.

The genesis of the clot in the ceca is not clear. The cases studied were not timed so as to encounter it in its formation. This gap must be filled before any basis for a discussion of the nature and significance of the cecal lesions can be found.

In general the larval stages of *Heterakis* were present but in small numbers. In four cases individuals were detected in the mucosa itself.

Flagellates so common in the tubules of the turkey were entirely absent in the two series of chickens.

The injurious effects of feeding embryonated eggs of *Heterakis papillosa* to young chickens are no less definite than those observed after feeding them to turkeys. There are, however, certain distinctions to be drawn between the disease in turkeys and in chickens. The turkeys were found much more susceptible since the feeding produced uniformly a severe disease probably fatal in all birds if those that were chloroformed had been allowed to live longer. The effect on the condition of the young chickens was slight and they would probably have all survived.

The lesions due to the feeding differ materially as regards the liver. In turkeys this organ is almost uniformly invaded by *Amœba meleagridis* and the resulting foci of multiplication lead to a destruction of a variable amount of liver tissue, often over 50 per cent. In chickens the invasion is so slight that when it does take place the resulting lesions are scarcely more than microscopic in size. The liver is not wholly immune, however, as spontaneous cases now and then prove.

As to the ceca, the lesions induced in chickens appear formidable enough, but they probably undergo speedy resolution and the destroyed mucosa is covered with epithelium in due time. The after effects of the partial destruction of the mucosa may be more serious and tend to interfere with the normal growth of the chicken. This destruction may be permanent and lead to a replacement of the mucosa by scar tissue, as shown in the two spontaneous cases described above. In general it may be assumed that injury to the ceca due to *Heterakis* and *Amœba meleagridis* is not uncommon and its causal relation to other pathological conditions of poultry may be far reaching. Not until this worm has been largely suppressed can the extent of the injury due to it be inferred in retrospect. The degree of injury inflicted appears to be largely a question of dosage. The more ova ingested the more widespread and intensive the lesions, whereas the ingestion of a few does not appear to be dangerous even to turkeys.

Indications of a tendency of the nematodes to encyst in the walls of the ceca, as observed by Letulle and Marotel[7] in a pheasant, were wholly absent in the cases examined.

The presence of *Amœba meleagridis* in chickens still in the brooder after they have been fed ova either free or still within fragments of

[7] Letulle, M., and Marotel, *Arch. parasitol.*, 1908, xii, 361.

worms incubated in physiological salt solution for 15 to 20 days at room temperature points to the presence of *Amœba meleagridis* in the cultures. This cannot be confirmed or disproved until some method other than feeding *Heterakis* eggs is found which will induce conditions favorable to the invasion of *Amœba meleagridis* into the walls of the ceca. So far it has been impossible to start the disease by feeding incubated feces or cultures from which the worms and ova had been removed. It should be stated that protozoa resembling amebæ and flagellates have been found in the cultures of ova fed and a study of these is now under way. The relation of *Heterakis papillosa* to typhlitis in turkeys and chickens and the slightly varying morphology of the invading protozoa present for consideration the possibility that the latter may not necessarily belong to one species.

CONCLUSIONS.

Feeding embryonated eggs of *Heterakis papillosa* to brooder chickens led to a disease of both ceca, characterized by the presence of a core consisting of fecal matter, coagulated blood, and emigrated cells from the mucosa. The walls of the ceca were thickened as a result of cell invasion and multiplication, invasion and multiplication of *Amœba meleagridis* or allied parasites, and more rarely hemorrhage and edema. The respective parts played by *Heterakis papillosa* and the protozoa in starting the lesions and the source of the protozoa remain to be defined. The invasion of the liver by the protozoa was insignificant.

EARLY CHANGES FOLLOWING THE INJECTION OF TUBERCLE BACILLI INTO THE METAPHYSIS OF THE LONG BONES OF ANIMALS.

By JEAN OLIVER, M.D.

(*From the Pathological Laboratory and the Laboratory of Surgical Pathology of the Medical School of Leland Stanford Junior University, San Francisco.*)

Plate 4.

(Received for publication, April 2, 1920.)

It has long been recognized that tuberculous infection of long bones originates and is in great part confined to the metaphysis and epiphysis. There is a marked contrast in this localization to the diffuse process which follows infection with pyogenic organisms, and it is not surprising that many conjectures have been made to explain the observed facts. Notable among these is the suggestion of Lexer,[1] whose anatomical studies led him to believe that embolic deposit of bacteria in the region of the metaphysis is favored by the course of the arteries. On the other hand, Ely[2] claims that peculiarities in the structure of the bone marrow more clearly explain the facts.

The following experiments were done to examine the latter factor. No attempt was made to infect the bone in a manner analogous to spontaneous infection in man, as our interest is concerned with the cellular reaction which follows the infection, rather than with the path of the infection. As an aid in the determination of the various cell types the animals were vitally stained.

In normal vitally stained animals the leucocytes and the megacaryocytes show no deposits of the dye. The fibroblasts are as a rule also free from dye, though in intensely stained animals a few fine granules are seen at either end of the nucleus. The cell that shows the vital stain in greatest amount is the reticulo-endothelial cell which lines the venous spaces of the marrow. The protoplasm of

[1] Lexer, E., *Arch. klin. Chir.*, 1904, lxxiii, 481.

[2] Ely, L. W., Joint tuberculosis, New York, 1911.

these stellate or triangularly shaped cells is crowded with bright blue granules of varying size, so that the nucleus may be entirely hidden. It is these cells which have been described as phagocytosing blood pigments, and as normally possessing a brown pigment.[3] The reaction of these cells to the vital stain has caused them to be included in the large group termed histiocytes,[4] or resting wandering cells.[5] They correspond roughly to the macrophages of Metchnikoff.

The histogenesis of the tubercle has been studied with the aid of vital staining by several investigators. Oppenheimer,[6] Goldmann,[7] and Evans, Bowman, and Winternitz[8] have used this method in following the development of the tubercle in the liver, and Joest and Emshoff[9] in the lymph glands. Kiyono mentions the bone marrow,[4] but gives no details of his observations. All these investigators agree that the early groups of epithelioid cells forming the miliary tubercle are made up of, or derived from the vitally stained histiocytes found in these organs.

Technique.

Rabbits were given 20 cc. of a 1 per cent solution of trypan blue intravenously, and 2 days later, a trephine opening[10] was made in the metaphysis of the tibia and one to two drops of a normal salt solution emulsion of bovine tubercle bacilli injected into the marrow. Similar experiments were made with guinea pigs and tubercle bacilli of the human type. After varying intervals, from 3 to 9 days, the animals were killed and portions of the bone removed and fixed in 10 per cent formaldehyde. After fixation the marrow was removed from the bone, embedded in paraffin, and sections were appropriately stained.

[3] Brass, H., and Weidenreich, F., *Arch. mikr. Anat., 1te Abt.*, 1913, lxxxii, 61.

[4] Kiyono, K., Die vitale Karminspeicherung, Jena, 1914.

[5] Tschaschin, S., *Folia hæmatol.*, 1913–14, xvii, 317.

[6] Oppenheimer, R., *Virchows Arch. path. Anat.*, 1908, cxciv, suppl., 254.

[7] Goldmann, E. E., Neue Untersuchungen über die Äussere und Innere Sekretion des gesunden und kranken Organismus in der "vitalen Färbung," Tübingen, 1912, 67.

[8] Evans, H. M., Bowman, F. B., and Winternitz, M. C., *J. Exp. Med.*, 1914, xix, 283.

[9] Joest, E., and Emshoff, E., *Virchows Arch. path. Anat.*, 1912, ccx, 188.

[10] The operation was performed under ether anesthesia.

In all the animals so treated it was possible to demonstrate either the bacilli, definite tubercles and giant cells, or diffuse caseation, depending on the length of time that the animal lived after infection. Guinea pigs proved more satisfactory for the early stages, as the bovine bacilli used in the experiments on rabbits were difficult to stain.

EXPERIMENTAL.

In our experiments the earliest changes were observed 3 days following the operation. No lesion could be seen in the gross, and but little change was observed in sections with a low magnification. Around the area of injection, which showed little or no evidence of trauma, the lymphoid cells were decreased in number, while the vitally stained reticulo-endothelial cells were more numerous than in the normal marrow at a distance from the trephine opening.

With a higher magnification it was seen that a large number of the leucocytes showed evidences of nuclear degeneration, while the reticulo-endothelial cells contained two or more nuclei, and instead of being stellate in shape as normally, were now rounded and lay free in the sinuses of the marrow (Fig. 1). No mitotic figures were observed in these cells but the increase in their number and the presence of more than one nucleus to a single protoplasmic body warrant the assumption of an active proliferation on their part.

Another appearance noted in these cells was the presence of clear areas free from dye granules which showed no coloration with the counterstain. These were interpreted as vacuoles, and in sections stained for tubercle bacilli it was possible to demonstrate in them one or more acid-fast bacilli. With an increase in the number of bacilli in the cell there was a corresponding decrease in the number of dye granules. Fig. 2 shows a cell filled with bacilli and having many large vacuoles and a few faded dye granules.

In somewhat later stages of the infection groups of vitally stained reticulo-endothelial cells were seen consisting of from ten to twenty cells (Fig. 3). These cells contained acid-fast bacilli and showed varying degrees of the changes described above. It was impossible to determine whether these cells had proliferated *in situ* or wandered to the point of infection. The impression that the latter is the case

is obtained from the appearance of the cells, though both methods of increase doubtless occur.

A few giant cells were seen in the sections. These had the typical morphology of the Langhans type (Fig. 4). In their clear protoplasm could be seen dye granules and vacuoles, which in suitably stained specimens showed acid-fast bacilli. In many instances the contours of the individual reticulo-endothelial cells forming them could still be made out.

No attempt was made to follow the tuberculous process in its further development. Several animals were allowed to live a month or until death. In all these animals extensive tubercular lesions were found in the diseased bones, consisting of broad areas of caseation surrounded by granulation tissue. In this granulation tissue were many reticulo-endothelial cells still showing dye granules.

The demonstration by Lexer of numerous anastomoses in the metaphyses of long bones, though it may explain the more frequent embolic deposit of bacteria in that region, does not explain the difference observed in the localization of a tuberculous process, as contrasted with the diffuse lesion seen in pyogenic infections.

The present experiments emphasize the view that the observed difference may be due to peculiarities in the structure of the infected tissue, the bone marrow, for this tissue in the metaphyses of long bones as contrasted with the fatty marrow of the diaphyses, is rich in the cells which are particularly concerned in the reaction to infection with tubercle bacilli.

I wish to express my thanks to Dr. L. W. Ely and Dr. F. E. Blaisdell for their aid in the operations on the animals.

EXPLANATION OF PLATE 4.

All the figures were drawn with the aid of a camera lucida. Bausch and Lomb ocular 1, objective $\frac{1}{12}$.

FIG. 1. Two reticulo-endothelial cells from the marrow of a vitally stained rabbit infected 4 days previously with bovine tubercle bacilli. The dye granules are of normal appearance, but each cell body contains two large nuclei. Carmine counterstain.

FIG. 2. Reticulo-endothelial cell from the marrow of a guinea pig 6 days after infection. Many vacuoles are seen and a large number of phagocytosed tubercle bacilli. The dye granules are few in number and pale. Stained with carbol-

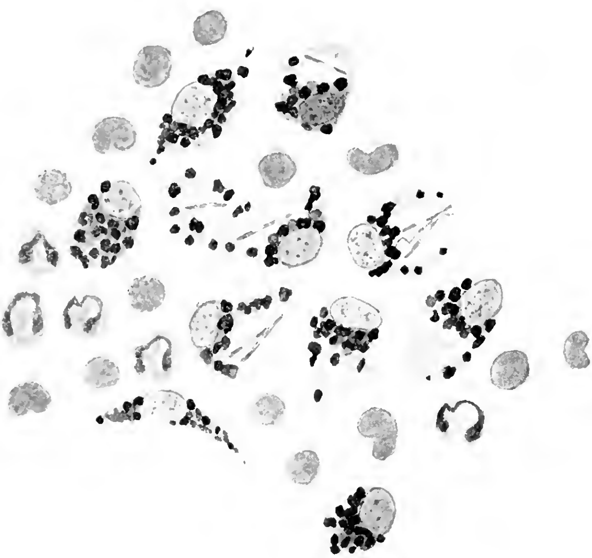
fuchsin, decolorized with acid alcohol, and counterstained with methylene blue.

FIG. 3. A group of vitally stained cells in the marrow of a guinea pig 9 days after infection. The cells show varying degrees of vacuolation and six of them contain tubercle bacilli. The same stain as in Fig. 2.

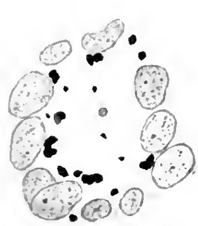
FIG. 4. The same specimen as Fig. 3. A typical Langhans giant cell with dye granules and vacuoles in the central protoplasm. Carmine counterstain.

1

2



3



4

(Oliver: Tubercle bacilli.)

IMPORTANCE OF BLOOD GROUPS IN COMPLEMENT FIXATION REACTIONS.

By WILLIAM C. WILLIAMS.

Captain, Sanitary Corps, U. S. Army.

(*From the Serological Laboratory of the Army Medical School, Washington.*)

(Received for publication, April 7, 1920.)

The complement fixation test for syphilis has become one of the most valuable and widely applied of diagnostic reactions, in spite of the empirical nature of several features of its technique. Increasing experience has eliminated several sources of technical error, and progress is being made toward standardization and greater reliability. One of the well known sources of error in the original Wassermann test is the possible presence of an excess of amboceptor in the form of natural anti-sheep hemolysin in human serum. That this excess of amboceptor is of importance seems to have been proved by many workers. The recent results obtained by Kolmer and Rule (1) are especially conclusive.

In this paper attention is called to a similar possible source of error with the use of an anti-human hemolytic system. It is shown that an excess of amboceptor may result from a natural hemolysin in the guinea pig complement and from the isolysins in human serum, when the blood cell suspensions are made up at random. A simple way of avoiding this possible inaccuracy is pointed out, which consists in the use of a certain human blood group in the preparation of the human cell suspensions. Blood groups among sheep seem to be less clear and less important than among human beings.

The remark has occasionally been made by different serologists that, theoretically, the human blood groups might be of importance in complement fixation work with the human system. Search of the literature, however, has failed to reveal any instance of a practical application of this theory. The only statement found which directly bears on this subject is in War Manual No. 6, Laboratory methods,

U. S. Army (2), which gives the theoretical possibilities but makes no mention of the actual importance of blood groups, and no practical application of this theory had been made in this laboratory until the present study was completed. In a recent article Kolmer, Trist, and Flick (3) report their results with human isolysins in serological work, but they make no effort to apply the results practically, and, from the standpoint of this paper, their experiments are not conclusive, for while they use several different cell suspensions against different human sera, no statement is made as to the blood groups to which each cell suspension belonged. That this is of major importance will be shown later.

As the work reported here was undertaken for practical information, the technique followed closely the method of Craig (4), which is in routine use at the Army Medical School. 0.1 cc. of the inactivated human serum was always used; the guinea pig sera and the pooled complement were always diluted to 40 per cent and the dose was expressed either in units or actual quantities. These quantities were held constant in all the work with an anti-human hemolytic system. The work with the anti-sheep hemolytic system was performed with the quantities, dilutions, and dosage used in what is commonly known as the quarter unit Wassermann test.

The human blood groups were designated according to the classification of Moss (5) as follows:

Group I. Serum agglutinates the cells of no other group.
Cells are agglutinated by sera of Groups II, III, and IV.
Group II. Serum agglutinates the cells of Groups I and III.
Cells are agglutinated by sera of Groups III and IV.
Group III. Serum agglutinates the cells of Groups I and II.
Cells are agglutinated by sera of Groups II and IV.
Group IV. Serum agglutinates the cells of Groups I, II, and III.
Cells are agglutinated by no other sera.

Moss (5), Grafe and Graham (6), Zinsser (7), and many others have made the observation that the isohemolysins and isohemagglutinins are practically parallel in the human blood groups. These observations have resulted in the formulation of a phrase which has become axiomatic in human blood group work preliminary to blood transfusion—hemolysis never occurs when agglutination is absent. It is believed that this phrase can be amplified to include the statement that it is always possible to obtain hemolysis when agglutination is present. In as far as this applies to the human blood groups only, the work reported here amply cor-

roborates the truth of both statements. Kolmer and his coworkers (3) state that the presence of hemagglutination and hemolysis bears no direct relation to each other. Their experiments are open to the same criticism as before, in that they were working with various sera and cells, the blood groups of which were unknown. It is possible that their failure to obtain hemolysis when hemagglutination was present was due to an insufficient amount of complement. The two instances in which they obtained hemolysis when no macroscopic agglutination was present is harder to explain and has never been encountered in our work with the human blood groups.

A failure to obtain hemolysis in group cells or by group sera, the groups of which had previously been determined by the agglutination method, was never encountered in this work, nor was hemolysis ever obtained when agglutination was absent. It is true, however, that, quantitatively, the hemolysin content of a human serum does not necessarily correspond to the agglutinin content. A serum giving strong agglutination for a certain group of cells may give slight or no noticeable hemolysis, in the quantity used, against the same cells. The hemolysin is present, however, and can always be demonstrated, when sufficient complement is present, by increasing the amount of serum.

It seems correct, therefore, to substitute the word "hemolyzes" for "agglutinates" in Moss' classification of the human blood groups; *i.e.*,

Group I.	Serum hemolyzes the cells of no other group.
	Cells are hemolyzed by sera of Groups II, III, and IV.
Group II.	Serum hemolyzes the cells of Groups I and III.
	Cells are hemolyzed by sera of Groups III and IV.
Group III.	Serum hemolyzes the cells of Groups I and II.
	Cells are hemolyzed by sera of Groups II and IV.
Group IV.	Serum hemolyzes the cells of Groups I, II, and III.
	Cells are hemolyzed by the sera of no other group.

Upon the above premises the work upon the influence of the human blood groups in complement fixation reactions was undertaken.

Presence of a Natural Specific Hemolysin in Guinea Pig Serum for Group I and Group II Human Cells.

Trouble has been experienced at various times in our complement fixation work by encountering complement that was hemolytic for the particular cell suspension being used. This was never a constant factor and would often be absent or at least not noticeable in the control tube containing complement and cells in the complement titration. The presence of this hemolysin was particularly noted in France while using an anti-sheep hemolytic system, and at one time became a serious problem, warranting the issuance of a special circular by the Director of the Division of Laboratories and Infectious Diseases warning all laboratories to be on the watch for it and suggesting that the serum of each guinea pig should be tested against the cell suspension used that day, before the sera were pooled for use as complement. Certain investigations were undertaken at this time under the direction of Major R. G. Hussey and with the benefit of occasional advice from Colonel Zinsser. The pressure of routine work was so great, however, that nothing definite was accomplished.

In some of the experimental work in France it was shown that the serum of these guinea pigs often contained hemolysins for both human and bovine cells, although these hemolysins were not constant and did not parallel the natural anti-sheep hemolysin present. With this fact in mind, since changing to the anti-human hemolytic system, I have tested out a portion of each guinea pig serum, before it was pooled as complement, against the human cell suspension being used that day. It was often observed and demonstrated that on certain days practically all the guinea pig serum would contain a sufficient quantity of anti-human hemolysin to render it unfit for use as complement. On these occasions many guinea pigs would have to be bled before it was possible to find three or four sera suitable for pooling and use as complement.

While engaged in some work with the four groups of human cells it was observed, in certain control tubes containing only complement and cells, that the tubes containing complement and Group I cells, and complement and Group II cells, showed a marked agglutination of the cells. The control tubes with Group III and Group IV cells

TABLE I.

Demonstration of a Natural Specific Hemolysin in Guinea Pig Serum against the Cells of the Human Groups I and II.

Guinea pig No.	Reaction with 0.1 cc. of a 5 per cent suspension of Group I cells.				Reaction with 0.1 cc. of a 5 per cent suspension of Group II cells.			
	Amount of 40 per cent dilution of guinea pig serum.				Amount of 40 per cent dilution of guinea pig serum.			
	0.1 cc.	0.2 cc.	0.3 cc.	0.4 cc.	0.1 cc.	0.2 cc.	0.3 cc.	0.4 cc.
1					−	+−	+−	+
2					−	−	+−	+
3					−	+−	+−	+
4					−	−	+−	+
5					−	+−	+	++
6					+−	+	++	++
7					−	+−	+	++
8					+	++	++	++
9					+−	+	++	++
10					−	−	+−	+−
11					+−	+−	+	+
12					−	−	−	+−
13					−	−	+−	+
14					+	++	++	++
15	−	+	+	++	−	+	+	+
16	+	++	++	++	+−	+	++	++
17	+−	+	++	++	+−	+	++	++
18	−	−	+−	+	−	−	−	+
19	−	−	+−	+	−	−	−	+−
20	+	++	++	++	++	++	++	++
21	+	++	++	++	+	++	++	++
22	+	++	++	++	+	++	++	++
23	++	++	++	++	++	++	++	++
24	+	++	++	++	+	++	++	++
25	+	+	++	++	+−	+	++	++
26	−	+−	+−	+	−	+−	+−	+
27	+	++	++	++	+	++	++	++
28	+−	+	+	++	+−	+	++	++
29	+−	+	++	++	+−	+	++	++
30	−	+−	+	+	−	+−	+−	+
31	+	++	++	++	+	++	++	++
32	++	++	++	++	+−	+	++	++
33	−	−	+−	+−	−	−	−	+−
34	+	++	++	++	+	++	++	++
35	+−	+	++	++	−	+−	+	++
36	+	++	++	++	+	++	++	++

++ indicates complete hemolysis; +, marked hemolysis; +−, slight hemolysis; −, no hemolysis.

TABLE I—*Concluded.*

Guinea pig No.	Reaction with 0.1 cc. of a 5 per cent suspension of Group I cells.				Reaction with 0.1 cc. of a 5 per cent suspension of Group II cells.			
	Amount of 40 per cent dilution of guinea pig serum.				Amount of 40 per cent dilution of guinea pig serum.			
	0.1 cc.	0.2 cc.	0.3 cc.	0.4 cc.	0.1 cc.	0.2 cc.	0.3 cc.	0.4 cc.
37	+−	+	++	++	+−	+	+	++
38	+−	+	++	++	−	+−	+	++
39	+−	+	++	++	−	+−	+	++
40	+	++	++	++	+−	+	++	++
41	+	++	++	++	+	++	++	++
42	+	++	++	++	+−	+	++	++
43	+	++	++	++	+−	+	+	+
44	+	++	++	++	+	++	++	++
45	+−	+−	+−	+	+−	+−	+−	+
46	+	++	++	++	+	++	++	++
47	+	++	++	++	+−	+	++	++
48	−	+−	+−	+	−	−	+−	+
49	+	++	++	++	−	+−	+	++
50	+−	+	++	++	−	−	−	+−
51	+−	+	++	++	−	−	+−	+
52	+	++	++	++	−	+−	++	++

showed no agglutination. On the assumption that when hemagglutinins were present, hemolysins could usually be demonstrated, a large number of guinea pigs was tested against different human cell suspensions of Groups I, II, III, and IV.

The guinea pig sera were diluted to 40 per cent, which is the strength of the complement as used in the Army Medical School, and increasing quantities, 0.1, 0.2, 0.3, and 0.4 cc., were set up against the cell suspensions of the four human groups. Larger quantities were not used, for the complement unit in the anti-human system generally falls in the neighborhood of 0.04 or 0.05 cc. Quantities as large as 0.2 cc. are seldom if ever used, and for practical purposes it was not considered necessary to use quantities larger than 0.4 cc. The results on over 50 guinea pigs were striking. No hemolysis whatever was obtained with any of the guinea pig sera with Group III and Group IV human cells. The results with Group I and Group II human cells are shown in Table I.

TABLE II.

Natural Hemolysin in Guinea Pig Serum before Absorption with Human Cells.

Guinea pig No.	Amount of 40 per cent dilution of guinea pig serum.	Reaction with 0.1 cc. of a 5 per cent suspension of cells of.			
		Group I.	Group II.	Group III.	Group IV.
	cc.				
53	0.1	+	+	–	–
	0.2	++	++	–	–
	0.3	++	++	–	–
	0.4	++	++	–	–

++ indicates complete hemolysis; +, marked hemolysis; + –, slight hemolysis; –, no hemolysis.

TABLE III.

Natural Hemolysin in Guinea Pig Serum after Absorption with Group I Human Cells.

Guinea pig No.	Amount of 40 per cent dilution of guinea pig serum.	Reaction with 0.1 cc. of a 5 per cent suspension of cells of.			
		Group I.	Group II.	Group III.	Group IV.
	cc.				
53	0.1	–	–	–	–
	0.2	–	–	–	–
	0.3	–	–	–	–
	0.4	–	–	–	–

++ indicates complete hemolysis; +, marked hemolysis; + –, slight hemolysis; –, no hemolysis.

TABLE IV.

Natural Hemolysin in Guinea Pig Serum after Absorption with Group II Human Cells.

Guinea pig No.	Amount of 40 per cent dilution of guinea pig serum.	Reaction with 0.1 cc. of a 5 per cent suspension of cells of.			
		Group I.	Group II.	Group III.	Group IV.
	cc.				
53	0.1	–	–	–	–
	0.2	–	–	–	–
	0.3	–	–	–	–
	0.4	–	–	–	–

++ indicates complete hemolysis; +, marked hemolysis; + –, slight hemolysis; –, no hemolysis.

TABLE V.

Natural Hemolysin in Guinea Pig Serum after Absorption with Group III Human Cells.

Guinea pig No.	Amount of 40 per cent dilution of guinea pig serum.	Reaction with 0.1 cc. of a 5 per cent suspension of cells of.			
		Group I.	Group II.	Group III.	Group IV.
	cc.				
53	0.1	+−	+	−	−
	0.2	++	++	−	−
	0.3	++	++	−	−
	0.4	++	++	−	−

++ indicates complete hemolysis; +, marked hemolysis; +−, slight hemolysis; −, no hemolysis.

TABLE VI.

Natural Hemolysin in Guinea Pig Serum after Absorption with Group IV Human Cells.

Guinea pig No.	Amount of 40 per cent dilution of guinea pig serum.	Reaction with 0.1 cc. of a 5 per cent suspension of cells of.			
		Group I.	Group II.	Group III.	Group IV.
	cc.				
53	0.1	+	+	−	−
	0.2	++	++	−	−
	0.3	++	++	−	−
	0.4	++	++	−	−

++ indicates complete hemolysis; +, marked hemolysis; +−, slight hemolysis; −, no hemolysis.

In view of the fact that these guinea pig sera in the same quantities were absolutely negative for Group III and Group IV cells, the results indicate that there is, apparently, a specific hemolysin in guinea pig serum, in quantities that would be a factor if used as complement selective against two groups of human red blood cells. No hemolysin is present for the other two groups of cells in quantities far in excess of any that might be used in complement fixation reactions with an anti-human hemolytic system.

A fact of some importance is that these examinations were made at different times over a period of several months, that the guinea pigs were obtained from various lots purchased in Pennsylvania, Virginia,

Maryland, and the District of Columbia, and that in all the work the different human group cells were not obtained from any one set of individuals but from a large number of people, the only requirement being that they fell within the proper blood group.

The specificity of this hemolysin was next determined by the absorption, in the cold, of many of the guinea pig sera with the different group cells. A specimen of guinea pig serum, 40 per cent, was tested against the four group cell suspensions. A portion of this serum was then treated with Group I cells, at freezing temperature, for ½ hour, rapidly centrifuged, the supernatant serum pipetted off and retested against all four group cell suspensions. Three other portions of the untreated serum were then absorbed with the cells of Groups II, III, and IV respectively, in the same manner, and all separately retested with the cell suspensions of all four groups. A detailed tabulation of the experiments will not be given here, but the results were invariably constant and sample protocols are shown in Tables II to VI.

These experiments show that the natural anti-human hemolysin present in guinea pig serum can be absorbed by the cells of either Group I or Group II, but is not absorbed by the cells of either Group III or Group IV, thus proving the specificity of the hemolysin. As stated before, these experiments were done many times with unvarying results.

Influence of the Isohemolysins in Human Sera upon Human Cell Suspensions.

The influence of the isolysins in sera received for Wassermann test and their relation to the blood group of the cell suspension used were next investigated.

Twenty-three sera, upon which tests had been run, were selected at random. Group I cells were first selected as being, theoretically, the cells most apt to be acted upon by the largest number of sera. 0.1 cc. of the inactivated serum, 0.1 cc. of the Group I cell suspension, and one unit of complement, as determined for the tests that day, were made up to a total volume of 1 cc. with saline solution and incubated in the water bath at 37°C. for 1 hour. Three of the sera gave complete hemolysis of the cells, nine gave marked hemolysis, six gave

slight hemolysis, while five gave no appreciable hemolysis at the end of 1 hour. The three sera that gave complete hemolysis with Group I cells were then set up with a suspension of Group IV human cells, with the same quantities and technique. No hemolysis took place

TABLE VII.

Influence of the Isohemolysins in Twenty Human Sera on the Four Groups of Human Cells.

Serum No. (0.1 cc.).	Amount of complement (40 per cent).	Reaction with 0.1 cc. of a 5 per cent suspension of cells of.			
		Group I.	Group II.	Group III.	Group IV.
913	1 unit.	++	+	+−	−
914	1 "	+	−	−	−
915	1 "	+	+−	−	−
916	1 "	+	+−	−	−
917	1 "	++	+−	−	−
918	1 "	+	−	+−	−
919	1 "	+	−	−	−
920	1 "	++	−	+−	−
921	1 "	+	−	−	−
927	1 "	+−	+−	+−	−
928	1 "	++	++	+	−
929	1 "	−	−	−	−
930	1 "	+	+	−	−
931	1 "	+	+−	+	−
932	1 "	++	−	++	−
933	1 "	+−	−	−	−
934	1 "	++	−	+	−
935	1 "	+	−	−	−
936	1 "	+	+−	−	−
937	1 "	+	+−	−	−
Controls.					
0	2 units.	A	A	−	−
0	0	−	−	−	−

++ indicates complete hemolysis; +, marked hemolysis; +−, slight hemolysis; −, no hemolysis; A, agglutination.

with the Group IV cell suspension. The results with Group I cells were so suggestive that steps were immediately taken to continue the work with cells from all four of the human blood groups.

Twenty sera were next tested with cell suspensions of all four groups. The results are shown in Table VII. Hemolysis, marked or partial,

took place with practically all the human sera and Group I cells. Variations were shown in their action on Group II and Group III cells, according to the blood group to which each serum belonged. The Group IV cell suspension was not hemolyzed by any of the sera.

TABLE VIII.

Influence of the Isohemolysins in Twenty-Six Human Sera When Complement from Which the Anti-Human Hemolysin Had Been Absorbed Was Used.

Serum No. (0.1 cc.).	Amount of complement (absorbed with Group II cells).	Reaction with 0.1 cc. of a 5 per cent suspension of cells of.			
		Group I.	Group II.	Group III.	Group IV.
1	1 unit.	+−	−	−	−
2	1 "	−	−	−	−
3	1 "	+	+−	+−	−
4	1 "	++	+	++	−
5	1 "	+−	−	+−	−
6	1 "	++	+−	++	−
7	1 "	+	−	+	−
8	1 "	+	−	+−	−
9	1 "	+−	+−	−	−
10	1 "	+	−	+−	−
11	1 "	+	+−	+−	−
12	1 "	+−	−	+	−
13	1 "	++	−	++	−
14	1 "	+−	+−	+−	−
15	1 "	+	+	+	−
16	1 "	+	+−	−	−
17	1 "	+−	−	−	−
18	1 "	+−	+	+−	−
19	1 "	−	−	−	−
20	1 "	++	+	+−	−
21	1 "	+	−	−	−
22	1 "	+	−	+−	−
23	1 "	+−	+−	−	−
24	1 "	++	+	+−	−
25	1 "	+−	−	+−	−
26	1 "	+	−	+−	−
Controls.					
0	2 units.	−	−	−	−
0	0	−	−	−	−

++ indicates complete hemolysis; +, marked hemolysis; +−, slight hemolysis; −, no hemolysis.

Although the one unit of complement used was a very small quantity (0.04 cc.) the possibility of the influence of the natural anti-human hemolysin in the complement against Group I and Group II cells could not be overlooked. The above experiment was repeated with complement from which the anti-human hemolysin had been previously absorbed. The complement was prepared in the usual way and was tested against all four groups of human cells. The natural hemolysin was then absorbed from it by treating it with Group II cells, at freezing temperature, for ½ hour. After absorption the complement was retested against all four group cell suspensions to verify the complete absorption of the hemolysin, in at least eight times the quantity used as one unit. Twenty-six sera were tested, as shown in Table VIII, the quantities used being the same as in the first experiment (Table VII).

The results obtained were similar to those shown in Table VII. With the natural hemolysin absorbed from the complement, hemolysis of some degree took place with practically all the sera and Group I cells, and with many of the sera and Group II and Group III cells, showing that the natural anti-human hemolysin present in the one unit of complement used in the first experiment was not entirely responsible for the hemolysis obtained with Group I and Group II cells.

The foregoing work has made it evident that, theoretically at least, the blood group to which the cell suspension belonged might play as important a part in the anti-human complement fixation reactions as the presence of natural anti-sheep amboceptor in human serum was supposed to play in the methods in which an anti-sheep hemolytic system was used.

Influence of an Excess of Amboceptor upon Complement Fixation Reactions in Which an Anti-Human Hemolytic System Is Used.

To determine the real practical importance of both the isolysins in human serum and the natural anti-human hemolysin in guinea pig serum, a series of complement fixation tests was done upon a number of sera which had given strongly positive, weakly positive, and doubtful reactions in the routine tests, all four group cells for each serum tested being used. Two units of complement were used, the

titer having been determined for use in the routine tests that day. The results are shown in Table IX.

Of the twenty-six sera tested there were but eleven that gave the same reacton with all four group cells, three being known negative

TABLE IX.

Complement Fixation Tests on Twenty-Six Human Sera against the Four Groups of Human Cells.

Serum No.	Reaction with a suspension of cells of.			
	Group I.	Group II.	Group III.	Group IV.
1	−	−	−	+−
2	−	+−	+−	+−
3	+	++	++	++
4	++	++	++	++
5	−	+−	+	++
6	−	+−	−	+−
7	+−	−	+−	+−
8	−	+−	+−	+−
9	++	++	++	++
10	++	++	++	++
11	++	++	++	++
12	+	+	+	++
13	++	++	++	++
14	+−	+	+	++
15	+	+	+	++
16	++	++	++	++
17	++	++	++	++
18	+	+	+	++
19	+−	+	+	+
20	−	−	−	−
21	+−	+	++	++
22	+−	++	+	++
23	+	++	++	++
24	++	++	++	++
25	−	−	−	−
26	−	−	−	−

++ indicates complete inhibition of hemolysis; +, partial inhibition of hemolysis; + −, slight inhibition of hemolysis; −, complete hemolysis.

Particular attention was paid to technique in these tests, all being run at the same time under identical conditions, the same reagents being used for all, and all steps being strictly controlled. Sera 20, 25, and 26 were known negative sera that were introduced as hemolytic and antigen controls.

sera introduced as controls. The fifteen sera showing variations, as a rule, gave the weakest reaction with Group I cells, but the results with Group II and Group III cells were sufficiently marked to show that the group of the cell suspension used plays an important part in the strength of the reaction obtained with the human hemolytic system.

TABLE X.

Complement Fixation Tests on Seventeen Human Sera against the Four Groups of Human Cells with Complement from Which the Natural Anti-Human Hemolysin Had Been Absorbed.

Serum No.	Reaction with a suspension of cells of.			
	Group I.	Group II.	Group III.	Group IV.
1	++	++	++	++
2	+−	+	+	++
3	++	++	++	++
4	+−	+	+	++
5	++	++	++	++
6	++	++	++	++
7	+	+	+	++
8	+−	+	+	+
9	++	++	++	++
10	−	−	−	−
11	−	+−	−	+−
12	−	−	−	+−
13	−	+−	−	+
14	−	+	+	+
15	+−	+	+−	+
16	+−	++	++	++
17	−	−	−	−

++ indicates complete inhibition of hemolysis; +, partial inhibition of hemolysis; +−, slight inhibition of hemolysis; −, complete hemolysis.

As the above tests had been done with the reagents and technique as ordinarily used in the routine complement fixation reactions, it was difficult to judge just how much of the variation in the reading was strictly due to the isolysins in the human sera and how much to the natural anti-human hemolysin present in the complement used. A second set of tests was run with various sera, all four groups of cell suspensions being used for each serum. The complement employed

had had the natural anti-human hemolysin absorbed from it. It was prepared in the usual way and tested against all four group cell suspensions. The natural hemolysin was then absorbed by treating the complement, at freezing temperature, with Group II human cells. After absorption the complement was retested against cell suspensions of all of the four groups to verify the complete absorption of the hemolysin. The unit of complement was determined by titrating against Group IV cells and two units were used in the tests. The same precautions as to technique, controls, etc., were observed here as with the first set of tests. Results were obtained as shown in Table X.

With the seventeen sera tested there were but seven that gave the same reaction with all four cell suspensions, five of these being strongly positive and two being known negative sera introduced as controls. With the ten remaining sera, weaker reactions were obtained with Group I, Group II, and Group III cells than were obtained with Group IV cells.

These results show that the isolysins in human serum are the important factor in the variations in the readings of the reaction obtained with the different group cell suspensions. The natural anti-human hemolysin in guinea pig serum may be a contributing factor to the resultant excess of amboceptor present when Group I or Group II cells are used, but it is of less importance, in the quantity ordinarily used in a two unit dose, than the human isolysins.

All the work so far points to the necessity, from the standpoint of accuracy, of using erythrocytes from a Group IV individual when preparing cell suspensions for work with a complement fixation reaction in which an anti-human hemolytic system is used. This is entirely compatible with our knowledge of the human blood groups, as Group IV cells are not acted upon by the isohemolysins in the serum of any of the human blood groups.

Human Blood Groups in Complement Fixation Reactions with an Anti-Sheep Hemolytic System.

Very early in the work reported above, the suggestion was made to the writer that the presence of natural anti-sheep hemolysin in human serum might bear some relation to the human blood groups. This could operate in two ways—either there might be certain of the

human blood groups whose sera contained hemolysin for all sheep cells, or, more possibly, there might be definite groups among sheep and it would perhaps be possible to select a sheep of a certain group whose cells might conceivably correspond to the human Group IV cells and contain no receptors for the hemolysins present in human serum. Such an observation as the latter, if it could be substantiated, would naturally be very important in complement fixation reactions with an anti-sheep hemolytic system.

TABLE XI.

Natural Anti-Sheep Hemolysin in Ten Human Sera Tested against Eleven Sheep Cell Suspensions.

Human serum (0.05 cc.).		Amount of complement (10 per cent).	Reaction with 0.25 cc. of a 5 per cent sheep cell suspension from.										
No.	Blood group.		Sheep A.	Sheep B.	Sheep C.	Sheep D.	Sheep E.	Sheep F.	Sheep G.	Sheep H.	Sheep I.	Sheep J.	Sheep K.
		cc.											
1	III	0.1	+	+	+−	+	−	++	−	+−	+−	+	+−
2	IV	0.1	++	++	++	++	++	++	++	++	++	++	++
3	III	0.1	−	+	−	+	+−	+	−	−	−	+	++
4	IV	0.1	++	++	++	++	++	++	++	++	++	++	++
5	III	0.1	++	++	++	++	++	+	++	++	+	++	++
6	IV	0.1	++	+−	+	++	++	++	++	+	++	++	++
7	II	0.1	++	+	+	++	+	++	+	+	+−	++	++
8	IV	0.1	+	+−	+	+	+	++	+	+	+	++	+
9	II	0.1	++	++	++	++	++	++	++	++	++	++	++
10	II	0.1	++	+	+	++	++	++	+	+	+	++	+

++, indicates complete hemolysis; +, marked hemolysis; +−, slight hemolysis; −, no hemolysis.

The problem of the effect of various human sera upon different sheep cells was approached by testing ten human sera, the blood group to which each belonged being known, against eleven different sheep cell suspensions. The technique used was that employed in the usual quarter unit Wassermann test. Inactivated human serum was used in a dose of 0.05 cc., complement was a 10 per cent dilution of pooled guinea pig serum, and the cell suspensions employed were all 5 per cent with a dosage of 0.25 cc. The complement was titrated against

a sheep cell suspension obtained from our own sheep, whose cells are not included in the eleven tested as shown in Table XI. The complement was also tested for hemolytic properties in a two unit dose against all of the eleven sheep cell suspensions used. The results are given in Table XI.

TABLE XII.

Natural Anti-Sheep Hemolysin in Sixteen Human Sera Tested against Twelve Sheep Cell Suspensions.

Human serum (0.05 cc.).		Amount of complement (10 per cent).	Reaction with 0.25 cc. of a 5 per cent sheep cell suspension from.											
No.	Blood group.		Sheep L.	Sheep M.	Sheep N.	Sheep O.	Sheep P.	Sheep Q.	Sheep R.	Sheep S.	Sheep T.	Sheep U.	Sheep V.	Sheep W.
		cc.												
11	III	0.1	+−	+−	−	+−	−	+	−	−	+−	+−	+	+−
12	IV	0.1	++	++	+	++	−	++	+	++	++	++	++	+
13	III	0.1	+	+	+−	+	−	+	+−	+	+	−	+	+
14	IV	0.1	++	+	+	+	−	++	+−	+	++	+	++	+
15	III	0.1	++	++	+	++	−	++	+	++	+	++	+−	++
16	IV	0.1	++	−	++	++	++	+	+	++	++	−	+	++
17	II	0.1	+	+−	−	+	+	+	+−	+	+	+−	+	+
18	IV	0.1	+−	++	++	+	+	+	−	+	+	+−	+	+−
19	II	0.1	++	++	++	++	++	++	+	+	++	++	++	+−
20	II	0.1	++	++	+	++	++	++	+	+	++	+	++	+
21	IV	0.1	−	−	−	−	−	−	−	−	−	−	−	−
22	IV	0.1	−	−	−	−	−	+	−	−	−	−	+	−
23	IV	0.1	−	−	−	−	−	−	−	−	−	−	+	+−
24	II	0.1	+−	++	++	++	−	++	++	++	++	++	++	++
25	IV	0.1	+−	+−	++	+	−	−	−	−	++	−	++	+
26	IV	0.1	++	++	++	++	−	++	++	++	++	++	++	++

++ indicates complete hemolysis; +, marked hemolysis; +−, slight hemolysis; −, no hemolysis.

The results obtained were so variable and irregular that the experiment was repeated. Human serum from sixteen different individuals, the blood group of each of whom was known, was used against twelve entirely different sheep cell suspensions. The technique employed was identical with that used above, and the same precautions were taken in testing and titrating the complement. The results are given in Table XII.

In Tables XI and XII are given the results on twenty-six different human sera, comprised in the commonest blood groups, ten of the sera being tested against eleven sheep cell suspensions and sixteen of the sera against twelve different sheep cell suspensions. All efforts to corelate these results with the various blood groups of the sera tested were futile. There appeared to be no regularity with which the serum from any particular blood group acted upon the cell suspensions.

On the other hand, there was a marked variation in the degree of hemolysis obtained with many of the individual sera against the different sheep cells. With certain sera this would vary from no hemolysis with one sheep cell suspension to complete hemolysis with another cell suspension, and almost all the human sera showed some variation in their action against the various cell suspensions. This fact is undoubtedly of some significance, although as can be seen, among the twenty-three sheep cell suspensions tested there was no single sheep whose cells failed to contain receptors for the anti-sheep hemolysin present in at least two or more of the human sera.

Further investigation along this line is being continued and these results, while unsatisfactory, are reported in order that other workers may investigate this variation in the susceptibility of different sheep cells to the natural anti-sheep hemolysin present in human serum.

Natural Anti-Sheep Hemolysin in Guinea Pig Serum.

Another problem to be considered was the occasional presence of natural anti-sheep hemolysin in guinea pig serum used as complement. That this may be of considerable importance in occasional instances has been shown above. During one period in which it was encountered in France approximately 150 guinea pigs from one lot had to be bled in order to obtain sufficient non-hemolytic serum that could be pooled and used as complement in the routine work. It occurred sporadically at that particular laboratory over a long period of time. It seemed strange, however, that during this period many competent observers in various subsidiary laboratories in France failed to find this hemolysin at any time in their guinea pig serum. At the time this was ascribed to a probable difference in the guinea pigs being

used, differences in breeding and inbreeding, as well as the possible influence of environment and food all being considered.

The results reported here would indicate that these views were probably erroneous and that the trouble was possibly due to a certain type or group of sheep cells for which certain guinea pig serum contains hemolysin, rather than to any inherent difference in the individual guinea pigs. This explains the failure of certain observers to confirm our observation, the particular sheep cells that they used not being susceptible to the action of, or not containing receptors for the hemolysin present in guinea pig serum. At the laboratory in question three sheep were in use most of the time, and so far as the writer knows, no strict rule was in force relative to the rotation of the bleedings. The sheep that was easiest to catch and hold was usually the sheep that was bled the most often.

When the work on the natural anti-human hemolysin in guinea pig serum showed that this hemolysin was selective for Group I and Group II human cells, it suggested the possibility of the presence of a natural anti-sheep hemolysin in guinea pigs which would only be active against a certain type or group of sheep cells. A large number of guinea pigs was tested against many sheep cell suspensions on different occasions. The guinea pig serum was used in a dilution of 10 per cent in amounts as indicated in Table XIII; cell suspensions were 5 per cent with a dose of 0.25 cc. In order to eliminate the possibility of anticomplementary action by any of the sheep cells, a hemolytic control was run on each cell suspension, with 0.2 cc. of the 10 per cent guinea pig serum and one unit of an artificial anti-sheep amboceptor. Detailed tabulation of the results need not be given, but a sample protocol of the test of four guinea pigs against seven sheep cell suspensions is shown in Table XIII.

In addition to the tests described above, twelve guinea pig sera against a total of fourteen different sheep cell suspensions gave negative results, which would indicate that it is a relatively uncommon condition for the average sheep cells to contain receptors for the natural hemolysins present in guinea pig serum in the quantities ordinarily used in complement fixation work. Table XIII, however, shows graphically what would happen if the sheep used for preparing the cell suspension should correspond to Sheep L. These results

would also explain the fact that a natural anti-sheep hemolysin was encountered with such frequency at one laboratory in France, while other observers failed to find this hemolysin in guinea pigs in use at other laboratories. The possibility of its occurrence, however slight, should be considered when selecting a sheep for complement fixation work.

TABLE XIII.

Natural Anti-Sheep Hemolysin in Guinea Pig Serum Tested against Seven Sheep Cell Suspensions.

Guinea pig No.	Amount of 10 per cent dilution of guinea pig serum.	Reaction with 0.25 cc. of a 5 per cent sheep cell suspension from.						
		Sheep L.	Sheep M.	Sheep N.	Sheep P.	Sheep R.	Sheep S.	Sheep W.
	cc.							
54	0.1	−	−	−	−	−	−	−
	0.2	+−	−	−	−	−	−	−
	0.3	+	−	−	−	−	−	−
	0.4	++	−	−	−	−	−	−
55	0.1	−	−	−	−	−	−	−
	0.2	+−	−	−	−	−	−	−
	0.3	+	−	−	−	−	−	−
	0.4	++	−	−	−	−	−	−
56	0.1	+	−	−	−	−	−	−
	0.2	++	−	−	−	−	−	−
	0.3	++	−	−	−	−	−	−
	0.4	++	−	−	−	−	−	−
57	0.1	+−	−	−	−	−	−	−
	0.2	+−	−	−	−	−	−	−
	0.3	+	−	−	−	−	−	−
	0.4	++	−	−	−	−	−	−

++ indicates complete hemolysis; +, marked hemolysis; +−, slight hemolysis; −, no hemolysis.

SUMMARY.

1. The presence of a natural anti-human hemolysin, only active against Group I and Group II cells, was demonstrated in over 50 guinea pigs, obtained from different sections of the surrounding country. It was shown that, in a majority of the sera tested, this

hemolysin would be a contributing factor to an excess of amboceptor in the reaction, in the quantities of complement used in an anti-human complement fixation reaction. The hemolysin is specific, in as far as it can be absorbed with either Group I or Group II cells, but cannot be absorbed with Group III or Group IV cells.

2. To determine the effect of the isohemolysins in human serum, twenty sera were tested against cell suspensions of the four human groups. Tests were also run with twenty-six human sera against all four group cell suspensions, with complement that had the natural anti-human hemolysin absorbed from it. In both experiments various degrees of hemolysis were obtained with all but two sera against cell suspensions from Groups I, II, and III, but no hemolysis was obtained with any serum against a Group IV cell suspension.

3. The importance of the human isohemolysins and the natural anti-human hemolysin in complement was demonstrated by performing complement fixation tests upon twenty-six human sera, cell suspensions of all four human groups for each serum tested being used. A marked variation in the reaction occurred with a large percentage of the sera tested. This variation in a single serum might be as great as a negative reading with Group I cells to a double plus reading with Group IV cells.

4. That the isohemolysins, alone, in human serum are of importance in the reaction was shown by running complement fixation tests upon seventeen human sera with the four group cell suspensions and complement from which the natural anti-human hemolysin had been absorbed. Results showed a much weaker reaction, in a large number of the sera, with Group I, Group II, and Group III cells than with Group IV cells.

5. Experiments to determine a relation between the human blood groups and natural anti-sheep hemolysin present in human serum gave negative results. It was shown, however, with twenty-six human sera tested against twenty-three different sheep cell suspensions that there is a wide variation in the activity of the natural anti-sheep amboceptor present in any human serum when it is tested against different sheep cell suspensions. A human serum capable of completely hemolyzing the cells of one sheep may fail to give any hemolysis whatever when tested against another sheep cell suspension.

6. Tests were made for natural anti-sheep hemolysin in guinea pig serum. The serum from sixteen different guinea pigs was tested against a total of twenty-one sheep cell suspensions. A natural anti-sheep hemolysin was present in all the guinea pigs tested against one of the sheep cell suspensions. The same guinea pigs tested against the other sheep cell suspensions failed to show any hemolysis.

CONCLUSIONS.

1. In the Wassermann reaction it should be taken into account that in addition to any complement-fixing bodies which may or may not be present, each serum tested belongs to a definite blood group and each human cell suspension used belongs to a definite blood group. These factors have never been sufficiently realized and must be taken into consideration if possible sources of error are to be excluded.

2. In addition to the isohemolysins in human serum, an additional source of error, when human cell suspensions are made up at random, is introduced by the presence of a natural anti-human hemolysin in guinea pig serum selective for Group I and Group II human cells.

3. Erythrocytes from a Group IV individual (Moss' classification) are never acted upon by the isohemolysins present in human serum nor are they acted upon by the natural anti-human hemolysin present in guinea pig serum.

4. Erythrocytes should always be obtained from a Group IV individual when preparing cell suspensions for complement fixation reactions in which an anti-human hemolytic system is used.

5. An unexplained variation in the activity of the natural anti-sheep hemolysin in human serum, when tested against different sheep cell suspensions, would seem to be worthy of further investigation.

6. The presence of a natural anti-sheep hemolysin in guinea pig serum used as complement, apparently only active against a certain type or group of sheep cells, is a factor to be considered when selecting a sheep for complement fixation work.

I wish to express my appreciation to Colonel F. F. Russell, Colonel C. F. Craig, and Major H. J. Nichols for helpful criticisms during the progress of this work.

BIBLIOGRAPHY.

1. Kolmer, J. A., and Rule, A., *Am. J. Syph.*, 1920, iv, 135.
2. Medical War Manual, No. 6, Laboratory methods of the United States Army, Philadelphia and New York, 2nd edition, 1919, 83.
3. Kolmer, J. A., Trist, M. E., and Flick, A. M., *Am. J. Syph.*, 1920, iv, 111.
4. Craig, C. F., The Wassermann test, St. Louis, 1918.
5. Moss, W. L., *Bull. Johns Hopkins Hosp.*, 1910, xxi, 63.
6. Grafe, E., and Graham, A. L., *Münch. med. Woch.*, 1911, lviii, 2257, 2338.
7. Zinsser, H., Infection and resistance, New York, 2nd edition, 1918.

EXPERIMENTS ON THE PRODUCTION OF SPECIFIC ANTISERA FOR INFECTIONS OF UNKNOWN CAUSE.

III. Nephrotoxins: Their Specificity as Demonstrated by the Method of Selective Absorption.

By GEORGE W. WILSON, M.D., and JEAN OLIVER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 5 to 7.

(Received for publication, April 16, 1920.)

The present study was undertaken as a continuation of work begun in this laboratory on the production of antisera for infections of unknown cause. It was found[1] that the infected tissues of animals might serve in some cases as an antigen for the production of immune sera, though with such antigens the sera inevitably contained antibodies injurious to the tissues of the animal. Experiments showed, however, that the sera can be largely deprived of these latter by incubation with successive portions of red cells without impairment of their protective value. Whether there are substances injurious to special organs which cannot so be taken out has remained to be determined. The problem is closely connected with that of the specificity of cytotoxins.

For most organs truly specific cytotoxins have not as yet been produced, despite many attempts. They have been elicited, though, for certain organs more or less isolated in the body. Zinsser sums up as follows on the subject:[2]

"Recent critical studies . . . . have revealed . . . . that the specificity of a serum produced with the tissues of one organ is not strictly limited to this organ alone, and that the serum may injure other organs as well. It is true, indeed, that there are certain cells and tissues in the body such as spermatozoa, the tissues of the testicles, the ovary, the lens of the eye, and possibly

---

[1] Rous, P., Robertson, O. H., and Oliver, J., *J. Exp. Med.*, 1919, xxix, 283.

[2] Zinsser, H., Infection and resistance, New York, 1914, 92.

the placenta, which have chemical characteristics so well defined and individual that the cytotoxic sera induced by them have definite organ specificity. The same to a more limited extent seems true of kidney substance (Pearce). In most cases, however, in which originally a specific cytotoxin was claimed, it has been possible to show subsequently that the apparently selective injury was not due to organ specificity alone but to the fact that the injection of tissue macerates, even when sufficiently freed from blood, induced the formation of considerable amounts of hemagglutinins and hemolysins."

In the light of such facts our problem resolves itself into a comparatively simple one: to determine whether one of the so called specific cytotoxins can be removed from a serum by exhausting the latter with red cells.

In selecting an organ for the study of "specific" cytotoxic sera the kidney seemed best adapted, because injuries to it can be determined by functional as well as histological methods. Pearce[3] has brought forward evidence that a serum can be produced which is, as he says, at last "special" in its action on the kidney. He leaves in doubt whether it can be termed specific in the strict sense, for despite the use of blood-free kidneys as antigen, his sera invariably contained hemolysins and hemagglutinins which might possibly account for their damaging effect on the kidney.

We have undertaken, first, to obtain a serum such as Pearce describes and observe its effects on the kidney; second, to determine whether the principle injurious to the kidney can be removed from this serum by absorption with red blood cells or with kidney tissue; and, third, to compare the efficacy of serum exhausted with red cells and that similarly treated with kidney tissue.

Production of Antikidney Serum.

Pearce's method was followed.[3]

Large dogs were etherized and under special precautions for asepsis their kidneys were removed and placed in glass boxes. The neighboring portion of the aorta was left attached to the kidneys in order to facilitate the passage of a cannula into the renal artery; and by prolonged washing with sterile salt solution the kidneys were practically freed of blood. The capsules were then stripped,

[3] Pearce, R. M., *J. Med. Research*, 1904, xii, 1.

the kidneys weighed and put through a meat grinder. The macerated tissue was pushed through a tea strainer of fine mesh with the aid of a pestle, small quantities of salt solution being added from time to time to facilitate its passage. The entire procedure was carried on under a glass plate surrounded by a canopy of sterile muslin which effectively protected the material from contamination.

The resulting heavy suspension in salt solution passed readily through a needle of medium sized bore. The material was usually injected into animals within 48 hours of its preparation, though occasionally it was kept in a frozen condition for some days prior to use. A number of rabbits received three to five intraperitoneal injections, each of from 4 to 8 gm. of kidney tissue, at intervals of 7 days, and they were bled on the 10th day after the last one. The sera were inactivated (56°C. for ½ hour) prior to use and their hemolytic and hemagglutinating titers tested, and cultures taken.

The sera without exception possessed moderately strong hemagglutinins and weak hemolysins for dog cells. Tests were made in the usual manner with graded dilutions in salt solution. Equal portions of the serum dilutions and of a 5 per cent suspension of thrice washed dog cells were used in the agglutination tests, and for hemolysis guinea pig complement in a dilution of 1 to 10 was added.

Precipitins were tested for but when found were negligible in amount.

Effects of Antikidney Serum.

Nine dogs were given a single intravenous injection of inactivated antikidney serum. The dose was 1 to 2 cc. per kilo of body weight. In most cases extremely marked renal changes resulted, differing considerably from those described by Pearce.

Prior to injection the general condition as well as the urine of the animals was carefully followed for a period of at least 2 weeks. Many supposedly normal dogs show albumin and casts in the urine without evident cause, and much delay was often experienced in finding animals suitable for our purpose. Within 24 to 48 hours after injection the animals appeared rather lethargic and gave the general impression of being sick. This period of depression passed off completely, or persisted, according to the potency of the serum and corresponding in a general way to the damage sustained by the kidney. The changes in the urine were marked. Severe albuminuria rapidly developed, with many casts, mostly granular at first but later many of them hyaline and occasionally waxy. This always occurred within 48 hours after the injection of the serum and persisted for the few days before the dog was killed. A trace of blood was sometimes noted.

With the exception of one dog (Dog C), which was observed for 4 months, all of the dogs were chloroformed and autopsied 10 to 14 days after injection. The kidneys were removed just prior to death or immediately after it. The viscera were examined, but no lesions were ever found that could be attributed to the serum. On two occasions more than 750 cc. of clear straw-colored fluid were present in the peritoneal cavity. No general edema was ever observed.

The kidneys showed pathological changes practically always, often of marked degree. In typical cases the organs were enlarged, and on stripping the capsule the kidney surface was everywhere mottled with bright red dots, from 0.1 to 1 mm. in diameter (Fig. 1, *A*). On section these were found to be the surface indices of hemorrhages into the cortex, the most characteristic gross finding. The hemorrhages were in general wedge-shaped, with the base of the wedge at the kidney surface. The glomeruli were indistinct and apparently had no relation to the hemorrhages. The cortex was swollen and opaque, and the pale medulla occasionally showed traces of hemorrhage extending from the cortex.

Histologically glomerular lesions, not the hemorrhages, were the most marked as well as constant finding. They occurred without hemorrhages when the serum was weak. In well marked instances there was an extensive necrosis in the glomerular tufts followed by obliterative hyaline changes (Figs. 2 and 3). A large proportion of the glomeruli were thus affected, though in even the most pronounced instances a sufficient number remained for kidney function. Accompanying the early cell changes was a plugging of the glomerular capillaries with fibrin and often a fibrinous exudation into the capsular space (Fig. 4). Desquamation and proliferation of the cells of the loops with occasional mitotic figures were observed. The end-result was often a complete obliteration of the glomerulus.

According to Pearce the most striking changes occur in the tubules. In our experiments lesions there were usually not pronounced, though sometimes marked cloudy swelling and desquamation were observed.

Next to the glomerular changes already described the most frequent findings were casts and hemorrhage into the tubules (Figs. 2 and 4). When the serum was strong small groups of a dozen to twenty tubules, usually in the neighborhood of a glomerulus, had their lumina filled

with blood cells (Fig. 5). These were the punctate hemorrhages so prominent in the gross specimen (Fig. 1).

There was also observable sometimes a considerable round cell infiltration between the tubules, especially in the neighborhood of the glomeruli. Some of the destroyed glomeruli showed a very heavy infiltration with lymphocytes and polymorphonuclear leucocytes (Fig. 6).

As a control two dogs were given large doses of normal rabbit serum. The results were negative as regards changes in the kidneys.

Effect of Antikidney Serum Exhausted with Red Cells.

The sera which caused the lesions described above contained, as already stated, hemolysins and hemagglutinins, though these were never strong. Steps were now taken to determine whether the action of the serum was due to them. Pearce performed a single experiment of the sort, submitting serum to contact with washed dog red cells for 1 hour at 0°C., and he found it thereafter "only faintly hemolytic." Kidney lesions were still produced. He records no observations, however, on hemagglutinins in the serum, which are often stronger and persist longer than hemolysins. In view of their ability to cause liver necroses,[3] such antibodies might well have been responsible for the lesions occurring in the kidneys.

By repeated absorptions with washed dog red blood cells, the technique of which has been previously described in detail,[1] our sera were completely deprived of hemolysins and hemagglutinins.

The results of the injection of dogs with serum thus treated are contrasted in Table I with findings in control animals receiving injections of corresponding doses of the same serum, untreated.

As the table shows, the exhaustion of the serum with red cells does not affect its ability to cause urinary changes. The albuminuria and cast formation were slightly less marked than in the controls. On the other hand, there were to the naked eye striking gross differences in the kidneys of the two series. It has been said that the untreated serum causes punctate hemorrhages in the renal cortex, often so numerous as to mottle diffusely the kidney surface. They are almost, or completely, lacking in animals given exhausted serum

TABLE I.

Effects of Nephrotoxic Sera, Untreated and Exhausted with Red Cells.

Experiment No.	Dog.	Serum No.	Treatment.	Serum titer. Action disappears at dilution of.	Dose per kilo of animal.	Effect on urine.		Killed after.	Condition of kidneys.
						Albumin.	Casts.		
					cc.			*days*	
1	A	1*	Untreated.	1/256 lysis. 1/128 agglutination.	1.55	+−	++	8	Slight gross changes; characteristic glomerular lesions and microscopic hemorrhages. Casts.
	Aa		11 cc. of serum underwent four absorptions with 1 cc. of red blood cells in all.	No lysins or agglutinins.	1.59	−	+	8	A few casts in tubules. Marked epithelial lesions.
2	B	2*	Untreated.	1/256 lysis. 1/128 agglutination.	1.33	−	+	8	Marked epithelial lesions. Casts.
	Ba		13.5 cc. of serum underwent four absorptions with 16 cc. of red blood cells.	No lysins or agglutinins.	1.72	+	−	8	Moderate epithelial lesions.
3	C	3	Untreated.	1/4 lysis. 1/1,024 agglutination.	1.65	++	+++	119	Few fibrotic glomeruli. No increase of interstitial tissue.
	Ca		17 cc. of serum underwent five absorptions with 25 cc. of red blood cells.	No lysins or agglutinins.	1.60	+++	+++	19	Severe glomerular lesions and hemorrhages. Many casts.

4	D	5	Untreated.	1/10 lysis. 1/30 agglutination.	0.60	++	++	9	Moderate glomerular lesions and hemorrhages. Many casts.
	Da		37 cc. of serum underwent five absorptions with 17 cc. of red blood cells.	No lysins or agglutinins.	1.36	++	+	9	Changes definite, but less than in the control.
5	E	5	Untreated.	As in Experiment 4.	1.85	++	++	10	Marked glomerular lesions. Hemorrhages and casts.
	Ea		Absorbed as in Experiment 4.	As in Experiment 4.	1.86	++	+	10	As in control, but slightly less marked.
6	F	6	Untreated.	1/4 lysis. 1/512 agglutination.	1.66	++	++	11	Numerous hemorrhages and glomerular lesions. Many casts.
	Fa		22 cc. of serum underwent seven absorptions with 22 cc. of red blood cells.	No lysins or agglutinins.	1.85	++	++	11	As in control.
7	G	7	Untreated.	1/128 lysis. 1/128 agglutination.	1.29	+++	++	10	Moderate changes of characteristic sort.
	Ga		15 cc. of serum underwent eleven absorptions with 34.5 cc. of red blood cells.	No lysins or agglutinins.	1.00	+++	++	10	As in control, but slightly fewer glomerular lesions.
	H		Untreated.	1/128 lysis. 1/128 agglutination.	0.65	++	++	11	Moderate changes of characteristic sort.
8	I	4	"	1/128 lysis. 1/16 agglutination.	1.65	+++	+++	22	Characteristic but moderate lesions.

* Sera 1 and 2 were kept 3 months before their absorption and the animal tests, which may explain the mildness of their effects.

(Fig. 1, *B*). To judge from the gross appearance one would suppose that exhaustion had entirely deprived the serum of its ability to cause renal lesions. Microscopically, however, it is seen that all of the lesions previously described are present save the hemorrhages. The glomerular and tubular lesions are in some instances less marked than in the controls, but in other cases they are equally well defined.

Type Experiment 1. Action of Nephrotoxic Serum. (*a*) *Untreated;* (*b*) *Absorbed with Red Cells.*—(*a*) Dog F, male, weight 6 kilos, after 2 weeks observation, during which time the urine was frequently examined and found to be free of casts and albumin, was given, on Mar. 26, 1918, 10 cc. of Serum 6 intravenously. The dog behaved normally after the injection. (This serum was prepared by injecting Rabbits 1 and 2 with blood-free dog kidney suspension. On Oct. 1, 1917, the suspension contained 4 gm. of kidney tissue; Oct. 8, 6 gm.; Oct. 15, 8 gm.; Oct. 22, 5 gm. Oct. 30. The rabbits were bled to death and the serum preserved in the ice box. On Mar. 22 the two sera were pooled and the titer was determined. Hemolysis disappeared at a dilution of 1 to 4 and agglutination at 1 in 512. Inactivation was done at 56°C. for ½ hour.)

Mar. 27. Urine very dark amber, alkaline, cloudy. Dense ring of albumin with nitric acid test. Many epithelial cells, a few red blood cells and leucocytes. No casts. Guaiac test positive. Mar. 28. Urine shows a great deal of albumin. Guaiac test positive. Occasional granular casts. Mar. 30. Same findings. Apr. 1. Albumin present. Guaiac test negative. Very many hyaline and granular casts. Apr. 3, 5, and 6 showed the same findings.

On Apr. 6 the dog was etherized and an autopsy performed, with negative findings in all the organs except the kidneys, which were swollen and congested. On stripping the capsule innumerable fine red dots up to 1 mm. in diameter were revealed on the surface of the kidney (Fig. 1, *A*). On section the kidneys were swollen and opaque, the cortex showing a definite widening.

Histological examination showed innumerable punctate cortical hemorrhages into the tubules, filling the lumina of scattered groups. There were a few interstitial hemorrhages. The majority of the glomeruli showed marked changes. Many of the cells in the tufts were necrotic, and numerous coils were plugged with fibrin. Mitoses were not infrequent. There was some interstitial round cell infiltration here and there between the tubules, especially in the neighborhood of the glomeruli. Many casts were seen in the tubules, but only slight changes in their epithelium.

(*b*) Dog Fa; weight 5.25 kilos. Observed 2 weeks, during which the urine was free from albumin and casts. The animal was given intravenously, on Mar. 26, 1918, 9.8 cc. of Serum 6, which had been absorbed six times with washed dog red blood cells. (The total amount of serum submitted to absorption was 22 cc. 3 cc. of washed dog red cells were added to it, the tube inverted several times and incubated at 37°C. for 1 hour. Dense agglutination resulted. After centrifu-

gation the serum was pipetted into another tube, another 3 cc. of washed cells were added, and incubation was repeated. In all, seven such absorptions were carried out on this serum, after which it was tested for hemolysins and agglutinins and found to possess none. Cultures taken prior to its use for injection proved that it was sterile.)

Mar. 27. Urine showed a trace of albumin, no casts. Guaiac test positive. Mar. 28. Same findings. Occasional granular casts. Mar. 30. Large amount of albumin, many granular casts, no blood. Apr. 3, 5, and 6. Same findings. Apr. 6. The dog was etherized and all organs found to be normal except the kidneys, which were large and pale. The capsules were stripped and revealed a few pin-point hemorrhages, probably one for every fifty seen in the case of Dog F (Fig. 1, *B*).

Histological examination revealed changes almost identical with those observed in the case of Dog F, except that there were fewer hemorrhages (Fig. 2).

Effect of Ordinary Hemolytic-Hemagglutinative Serum.

In view of the fact that hemolysins have far more effect *in vivo* than *in vitro*,[4] it seemed necessary to test the effect on the kidney of an ordinary hemolytic and hemagglutinating serum produced by the injection of dog red cells. For it might be contended, as explaining the injurious effect on the kidney of our exhausted serum, that such serum still contained traces of hemolysin and hemagglutinin, which, while not demonstrable *in vitro*, were active *in vivo*. Rabbits were immunized therefore against washed dog red cells and an anti-dog serum of high agglutinin and hemolytic titer was produced. This was inactivated and injected intravenously into dogs, a part of it after absorption with dog red cells.

Type Experiment 2. Effect of Hemolytic and Hemagglutinating Serum. (*a*) *Untreated;* (*b*) *Absorbed with Red Cells.*—The serum used was prepared by injecting rabbits intraperitoneally with a 20 per cent suspension of thrice washed dog cells in salt solution. Five injections were made at 7 day intervals, and the rabbits were bled on the 8th day following the last injection. The resulting sera were pooled and found to be hemolytic in dilutions up to 1 in 256 and agglutinative in dilutions up to 1 in 1,025. A portion of the pooled serum was subjected to eleven absorptions with dog red cells and again tested, with the result that no hemolysins or agglutinins were found. In detail the method of absorption was as follows:

[4] Muir, R., and M'Nee, J. W., *J. Path. and Bacteriol.*, 1911–12, xvi, 410.

Absorption.	Amount of serum.	Dog red cells.	Incubation.
	cc.	*cc.*	*min.*
1	20	3	30
2		8	30
3		3.5	40
4–11		4 each.	60–120

(*a*) Dog R, weight 5.5 kilos, was given, on May 6, 1918, 2 cc. of the untreated portion of the serum. May 8. The urine, previously normal, is now smoky in appearance, containing a trace of albumin and much hemoglobin but no casts. May 10. Urine dark red; faint trace of albumin; many granular casts containing brownish pigment. Tests for blood and bile positive. May 14. Urine dark amber; albumin negative; no casts. Bile pigment and blood present. May 17. Urine amber, no albumin, hemoglobin, or casts. May 18. Dog etherized and kidneys removed. Histological examination showed much brown, granular pigment in the spleen and liver. The kidneys were entirely normal except for widespread, fairly abundant, light brown pigment granules in the cells of the proximal convoluted tubules.

(*b*) Dog S, weight 5.25 kilos, was given intravenously on May 2, 1918, 12 cc. of the absorbed portion of the serum. Immediately after the injection dog vomited, was prostrated, and passed feces. Complete recovery took place in 1 hour. The urine was examined on May 3, 4, 6, 8, 10, and 14, but on none of these occasions were any abnormal constituents found. On May 14 the dog was etherized. Autopsy showed all organs normal. On histological examination the kidneys were normal.

It will be seen that the unabsorbed serum produced no important anatomical changes in the kidney despite the marked blood destruction it caused. Pearce, using such a serum, reports a fatty condition limited almost entirely to the loops of Henle. Otherwise the urinary and kidney findings which he reports in animals that survived a few days were the same as here reported. He did not try absorption, which, as we found, renders the serum innocuous to the blood as well as the kidneys. Altogether the facts warrant the conclusion that the changes produced by an antikidney serum exhausted with red cells cannot be attributed to persisting hemolysins or agglutinins. It is interesting that the most striking gross lesion caused by the antikidney serum, namely the punctate cortical hemorrhages, is not produced by an anti-red-cell-serum, although absorption with red cells deprives the antikidney serum of its ability to produce such a lesion.

Effects of the Exhaustion of the Serum with Kidney Tissue.

Can the injurious principle in the antikidney serum be removed by absorption with kidney tissue? This point was now investigated.

For the purpose of proper absorption with kidney tissue it was obviously necessary that the latter should be in as finely divided a state as possible, so as nearly to resemble in amount of absorbing surface a red cell suspension. Coarse fragments of tissue would offer relatively little surface and might be expected to yield poor results.

To obtain a fine suspension the tissues of washed kidneys of dogs were ground in the way already described, taken up in normal salt solution, and shaken with broken glass for 1 hour. The glass and larger tissue fragments were then removed by slow centrifugation, leaving a dense, milky suspension of finely divided material. On microscopic examination many free kidney cells were found in this fluid, which was now sedimented at high speed, and the sediment repeatedly washed by centrifugation until the supernatant fluid came away perfectly clear. The sediment was found to consist of parenchymal and other cells and fragments of them. For the purpose of absorption it was measured in bulk and used in suspension in the same manner as blood cells. It went into suspension readily.

Type Experiment 3 (Table II). Inactivated Antikidney Serum X Was Divided into three Portions: (a) Untreated; (b) Absorbed with Red Blood Cells; (c) Absorbed with Different Amounts of Kidney Tissue.—The untreated serum weakly agglutinated dog red cells in a dilution of 1 to 32 and hemolyzed them completely in a dilution of 1 to 4.

(*a*) *Results with Untreated Serum.*—Dog U, weight 7 kilos, with a urine negative for albumin and casts, received 1.35 cc. per kilo of the untreated serum on June 11, 1918. No symptoms following the injection. The urine on June 14, 19, 21, and 22 showed a heavy cloud of albumin and many hyaline and granular casts.

On June 22 the dog was etherized and an immediate autopsy performed. All organs were negative except the kidneys, which were swollen and congested. A few punctate hemorrhages were seen on the surface.

Histological Examination.—All organs negative except the kidneys, which showed characteristic changes. Many of the glomeruli were greatly damaged, though not a majority. All showed increase of cell nuclei, and the loops were often difficult to distinguish and appeared collapsed, apparently as a result of small occluding fibrin masses. There were some glomerular adhesions. The proximal convoluted tubules were definitely swollen, with much albuminous debris in the lumina, as well as many casts. The lumina of the descending portion of the loop of Henle were filled with red cells in places. There were small, scattered areas of edema of the interstitial tissues. The liver and spleen showed nothing abnormal.

(*b*) *Results with Serum Absorbed with Red Cells.*—Dog V, weight 8.5 kilos, received intravenously, on June 11, 1918, 1.28 cc. per kilo of Serum X, absorbed with dog red blood cells. The following was the method of absorption.

Absorption.	Amount of serum.	Dog red cells.	Incubation.
	cc.	*cc.*	*min.*
1	22	6	50
2		3.5	60
3–4		3 each.	60 and 90

After the absorptions there were no demonstrable hemolysins or agglutinins in this serum.) No symptoms. Urine negative.

June 13. Urine negative. June 14. Albumin, dense ring. No casts or blood. June 19 and 21. Urine showed much albumin, few granular and hyaline casts. On June 21 the dog was etherized. All organs were found negative with the exception of the kidneys, which were congested and swollen; no surface hemorrhages.

Histological Examination.—All organs negative with the exception of the kidneys, which showed a few interstitial hemorrhages. The usual glomerular changes were present. Few casts seen in the tubules. Findings like those of Dog U but not quite so pronounced.

Dog W, weight 4 kilos, received intravenously, on July 13, 1918, 4.4 cc. of Serum X, absorbed with red cells as above. July 15. Urine contained much albumin, occasional granular casts, no blood. July 17, 20, 22, and 24. The urine contained much albumin, many granular casts, no blood. Dog etherized and kidneys removed on July 24. Autopsy showed all organs normal with the exception of the kidneys, which were large and pale, with a very few punctate hemorrhages on their surface.

Histological Examination.—The general picture was the same as that of Dog U.

(*c*) *Results with Serum Absorbed with Kidney Cells.*—Dog X, weight 6.25 kilos, received, on June 11, 1918, 8 cc. of Serum X, absorbed three times with kidney cells in exactly the same proportion and for the same period employed with red cells as above stated. No symptoms. Urine examined June 13, 14, 19, 21, and 22. With the exception of June 14, when a very slight trace of albumin was observed, it was negative throughout. Etherization and autopsy on June 22. All organs normal by gross and histological examination.

Dog Y, weight 5.75 kilos, received, July 13, 1918, 8 cc. of Serum X, which had been absorbed three times with washed kidney cells of dogs as just described. No symptoms. Urine showed slight traces of albumin and occasional granular casts on July 15 and 17. On July 20, 22, and 24 it was negative. July 24. The dog was etherized and autopsied. Kidneys small, slightly congested, otherwise normal. All other organs normal.

Histological Examination.—Negative.

Dog Z, weight 4.50 kilos, received, on June 27, 1918, 2.2 cc. per kilo of Serum X, absorbed with kidney tissue as described. There followed a severe reaction, with vomiting, rapid respiration, and passage of feces and urine. The general picture suggested an anaphylactic crisis. After 20 minutes there was a gradual cessation of these symptoms, with complete recovery.

July 1. General condition of dog good. Urine showed albumin and a few granular casts but no blood. July 3. Urine about the same. July 5. Many casts.

TABLE II.

Relative Effects of Untreated Antikidney Serum and Serum Exhausted with Red Cells and with Kidney Cells (Type Experiment 3).

Dog.	Serum X.	Total bulk of sediment used for exhaustion per cc. of serum.	Dose of serum per kilo of animal.	Urine changes.	Kidney changes.
		cc.	*cc.*		
U	Untreated.		1.35	++	Moderate lesions in epithelium and glomeruli.
V	Four absorptions with red blood cells. Total incubation 5 hrs.	0.70	1.28	++	Slight glomerular lesions; moderate epithelial.
W	" "	0.70	1.10	++	Moderate glomerular lesions; no hemorrhages. Many casts.
Q	" "	0.70	2.17	++	Severe glomerular lesions; epithelial injury. Casts.
X	Three absorptions with kidney cells. Incubation 4 hrs.	0.50	1.26	0	Occasional cast. Otherwise negative.
Y	" "	0.68	1.39	0	Occasional cast.
Z	" "	1.00	2.2	+	Rare glomerular lesion. Few casts.

July 8. Only a trace of albumin, few casts. Dog etherized and autopsied. General examination negative. Kidneys showed no gross changes except a few hemorrhages, very few compared with control.

Histological Examination.—Occasional glomerular changes of the sort already described, but on the whole most of the glomeruli were in excellent condition. There were not infrequent characteristic hemorrhages into groups of tubules, as well as interstitial hemorrhages. Tubules generally normal, with the exception of a few casts in lumina. Liver and spleen normal.

The results of the experiment are summarized in Table II.

DISCUSSION.

Pearce's conclusion that an anti-dog rabbit serum could be produced which has a special action on the kidney has been confirmed. He, however, left the question of the true specificity of such a serum in doubt, and we must now consider whether our experiments throw any light on the subject.

An element in antikidney serum causing a very striking lesion, (abundant punctate cortical hemorrhages) is removed from the serum by the exhaustion of the latter with red cells, although an ordinary anti-red-cell-serum fails to produce such lesions. But the principle most injurious to the kidney cannot be so removed, even when the number of absorptions and the total bulk of red cells are very large. Moreover, a serum of high titer obtained by immunization with washed red cells fails to produce kidney lesions in any way resembling those of the nephrotoxic serum, which is further evidence that the injurious principle of the latter is not an hemagglutinin or hemolysin. The absorption of the antikidney serum with kidney tissue removes the injurious antibodies, even when the amount of tissue employed, and presumably the absorbing surface, are much less than were used in similar absorptions with red blood cells. Illustrations of the point are to be found in Table II.

The criticism may be made that the great absorptive power of the kidney tissue as thus demonstrated need not be the consequence of specificity but may be related to physical conditions that obtain in the emulsion, which make its absorptive power greater than that of a red cell emulsion. The experiments of certain observers on the absorption of antibodies with substances which obviously have no specific relation to them, kaolin, for instance, might here be cited. This criticism, however, is primarily directed not at the specificity of the nephrotoxin, with which we are alone concerned, but at the basic theory of the specificity of antibodies in general.

The effects of the nephrotoxic serum are exerted on the glomeruli and to a somewhat less degree on the tubules. It may be asked whether a vascular lesion will not account for all of the results. The glomerular changes are not improbably secondary to an occlusion of the coils following injury to the vascular endothelium. Our ani-

mals were autopsied too late to furnish conclusive evidence on the point. But whether or not the primary lesion is vascular, we are certain at least that the kidneys alone are affected. Were this the result of a non-specific endotheliolysin other organs should serve equally as well as the kidney for an antigen because of their content in endothelial cells. Pearce, however, has shown that a serum produced by the use of liver tissue as antigen has no effect on the kidneys.

As has been stated, our investigation was begun with a view to determining whether an antiserum produced as a result of the injection of tissues of an infected organ can be freed of injurious tissue antibodies by its exhaustion with red cells. As far as the kidney is concerned the question has been answered in the negative. A serum produced by immunization with kidney tissue should be absorbed with material from this organ. When that has been done it is no longer injurious to the kidney. Whether these principles are of general application may be doubted, for it is admitted that the kidney constitutes a special case. Notwithstanding numerous attempts by various investigators, sera specific for the spleen, pancreas, and liver have never been conclusively demonstrated. To obviate all possibility of injury from specific antibodies, however, a serum produced by the use of a tissue antigen should be exhausted with tissue of an identical sort prior to its introduction into the animal body.

SUMMARY.

As Pearce has shown, a serum highly injurious to the kidney of dogs can be produced by the immunization of rabbits with washed renal tissue of the dog. The histological findings are striking and characteristic, the most noteworthy being a glomerular lesion of special type. The renal changes differ much from those Pearce described.

The injury to the kidney is not to be explained by hemolytic and hemagglutinative elements in the serum. The complete removal of such antibodies by exhaustion of the serum with successive portions of red cells fails to lessen materially its ability to cause kidney lesions. Furthermore, an ordinary hemolytic and hemagglutinative serum produced by the use of washed red cells as antigen fails to cause similar lesions.

The distinctive, injurious principle of antikidney serum can be removed and the latter rendered innocuous by absorption with kidney tissue. To all practical intents and purposes it would seem that nephrotoxic serum of the sort here described is specific.

If infected tissue is to be utilized as an antigen for the production of therapeutic antisera the latter must in some instances be exhausted with tissue of the same sort prior to introduction into the animal body.

EXPLANATION OF PLATES.

Plate 5.

Fig. 1. Photograph of the kidneys of Dogs F and Fa, Type Experiment 1, Table I. (*A*) The first mentioned animal received untreated antikidney rabbit serum, and its kidney shows innumerable cortical hemorrhages. (*B*) The other received an even larger amount of the same serum, from which the hemolysins and hemagglutinins had been removed by repeated absorptions with dog red cells. Though the kidney shows almost no hemorrhages, it was found microscopically to be badly damaged (Fig. 2).

Fig. 2. Section of the kidney pictured in Fig. 1 of Dog Fa, Type Experiment 1. Three glomeruli are seen, all of which show necroses of varying size. The tubules are dilated and filled with hyaline casts. There is beginning round cell infiltration in the neighborhood of the glomeruli. Bausch and Lomb, obj. $\frac{2}{3}$, oc. 1.

Plate 6.

Fig. 3. Section from Dog Q, Type Experiment 3, which received antikidney serum absorbed with red cells. High power view of a necrotic glomerulus. Half of the glomerulus is entirely destroyed, and the other half severely compressed by the necrotic mass There is a beginning invasion of the dead material by proliferating capsular cells. Bausch and Lomb obj. $\frac{1}{6}$, oc. 1.

Fig. 4. Same animal. Weigert fibrin stain of an injured glomerulus. The necrotic area contains a large thrombus of fibrin. Three tubules are also seen filled with hyaline casts which take the fibrin stain. Bausch and Lomb obj. $\frac{2}{3}$, oc. 1.

Plate 7.

Fig. 5. Same animal. A group of tubules, lying just beneath the capsule, their lumina filled with red blood cells. Other tubules contain hyaline casts. Bausch and Lomb obj. $\frac{2}{3}$, oc. 1.

Fig. 6. Same animal. An almost completely destroyed glomerulus. There is a marked infiltration of the glomerulus and the surrounding area with leucocytes, both polymorphonuclear and lymphocytic. Bausch and Lomb obj. $\frac{2}{3}$, oc. 1.

A B

FIG. 1.

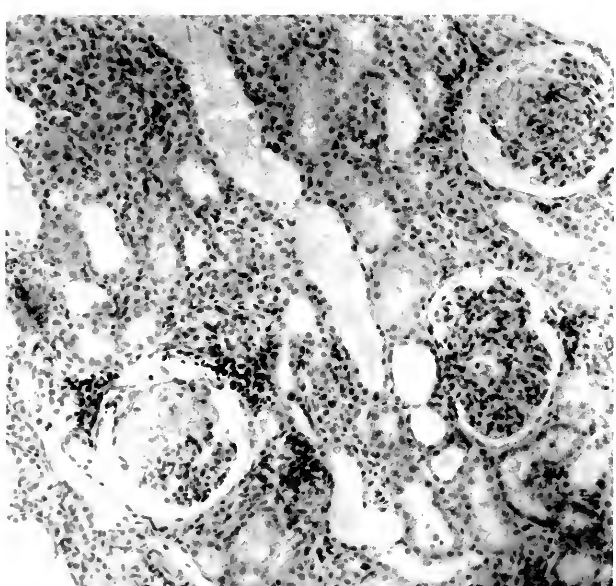


FIG. 2.

(Wilson and Oliver: Production of specific antisera. III.)

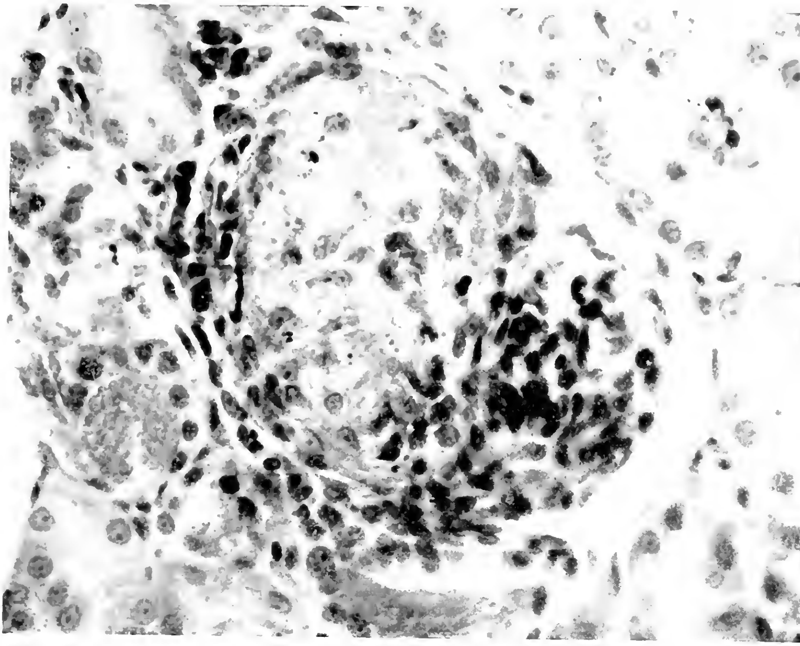


FIG. 3.

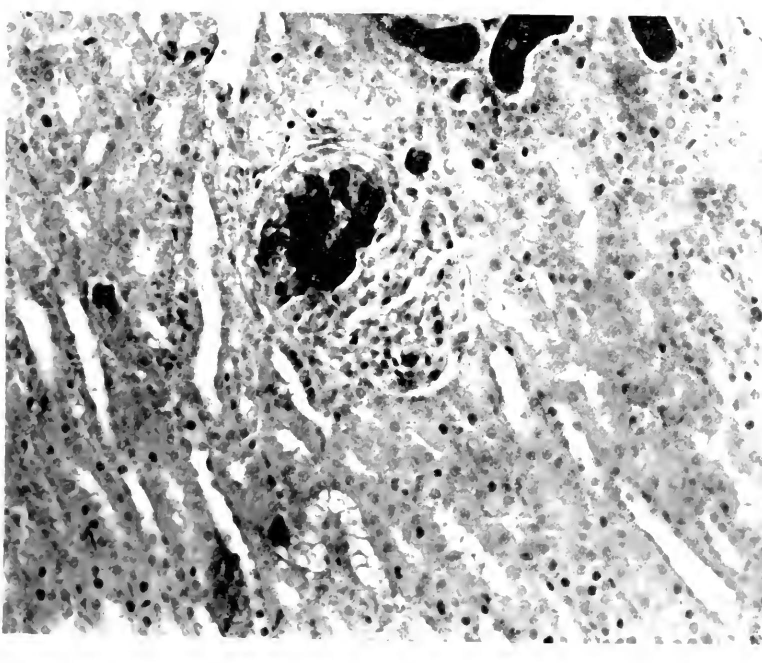


FIG. 4.

(Wilson and Oliver: Production of specific antisera. III.)

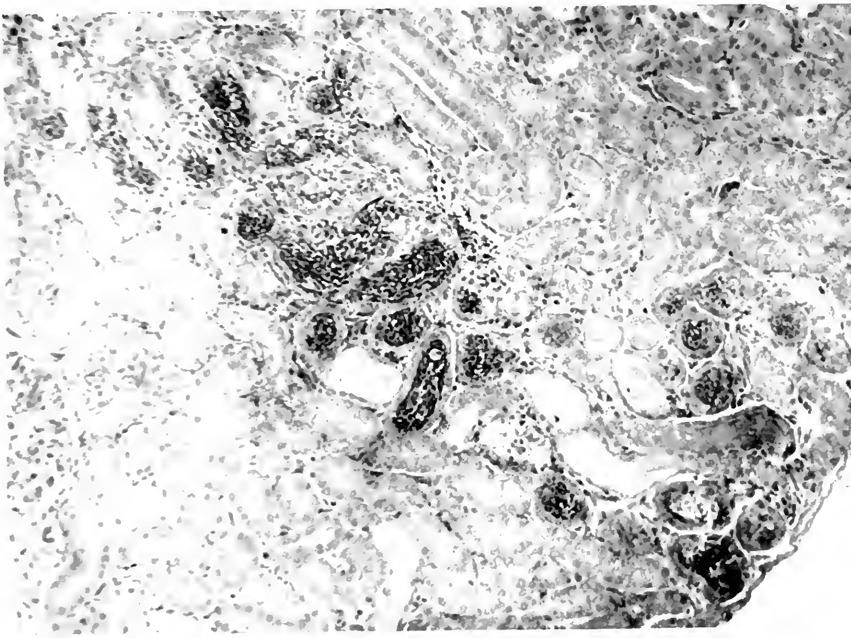


FIG. 5.

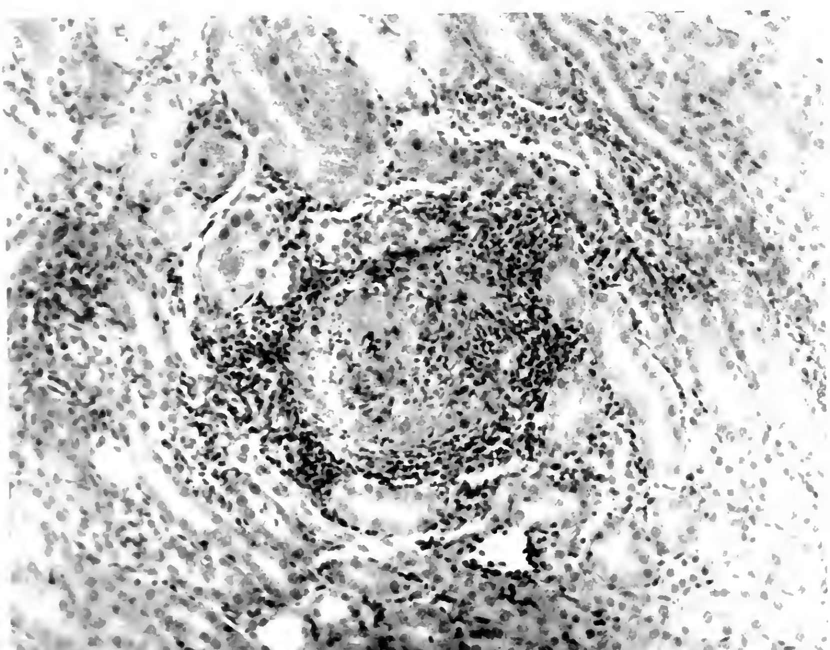


FIG. 6.

(Wilson and Oliver: Production of specific antisera. III.)

EFFECT OF INTRABRONCHIAL INSUFFLATION OF ACID.

By M. C. WINTERNITZ, M.D., G. H. SMITH, Ph.D., and F. P. McNAMARA, M.D.

(*From the Brady Laboratory of Pathology and Bacteriology of the School of Medicine, Yale University, New Haven.*)

(Received for publication, March 31, 1920.)

The resemblance between the lesions of influenzal pneumonia[1] and those produced by the inhalation of pulmonary irritating gases[2] suggested a series of experiments which might assist in the interpretation of the characteristic influenzal lesions. Many of the war gases, particularly those which act as pulmonary irritants, contain free chlorine or liberate it when they are decomposed. The weight of evidence[3] also indicates that the lesions which these gases produce depend in part upon the halogen radical, and that variations in the lesions correspond with variations in the halogen concentration as well as with the portion of the respiratory tract attacked. Moreover, it is conceded that the decomposition of these gases is associated with the formation of hydrochloric acid. The experiments now reported show that intrabronchial insufflation of hydrochloric acid solutions causes similar lesions, and these are also identical with the lesions disclosed in the examination of the respiratory organs of individuals who die of influenza.

EXPERIMENTAL.

At first isotonic saline solution with hydrochloric acid added to a strength of 1 per cent was used. By intrabronchial insufflation 5 cc. of this solution were forced into the pulmonary alveoli of rabbits previously anesthetized completely. Immediately a blood-stained,

[1] Winternitz, M. C., McNamara, F. P., and Wason, I. M., Pathology of influenza, New Haven, 1920.

[2] Winternitz, M. C., et al., Collected studies on the pathology of war gas poisoning, New Haven, 1920.

[3] Lillie, R. S., Clowes, G. H. A., and Chambers, R., *J. Pharmacol. and Exp. Therap.*, 1919, xiv, 75.

frothy fluid appeared at the mouth and nostrils, and as the catheter was removed convulsions developed. Death followed within 3 to 5 minutes.

At autopsy the very voluminous lungs filled the pleural cavities and covered the cardiac area. Occasionally the most affected lobe was collapsed, the remainder being voluminous. The glistening pink or pinkish gray pleural surfaces were tense and frequently showed hemorrhages which varied in size and shape. While all the pulmonary tissue was increased in volume, usually the most marked increase was localized on one side, even in a single lobe made conspicuous by the pleural hemorrhages. On section the lungs poured forth a large quantity of straw-colored or blood-stained fluid. After the fluid was washed away hemorrhages similar to those noted upon the surface were found within the pulmonary tissue. Frequently the peribronchial and perivascular sheaths were prominent—translucent, watery, gray, and thickened. The trachea and the larger bronchi contained blood-stained fluid, and hemorrhage into the mucosa was common. Histologically, as might be expected, there was little change. Death occurred too quickly to permit anatomical manifestation of the disturbance in the balance of cell function. There were a thick, colloid-like precipitate within the alveoli and edema of the interstitial, periarteriolar, peribronchial, and perivascular structures. There were also hemorrhages which involved both the pleural interstices, the perivascular and peribronchial tissues, and, in some instances, the alveoli. Edema followed insufflation of the hydrochloric acid solution much more quickly than it follows the inhalation of chlorine; otherwise the pathological changes were identical.

The acute cause of death after the insufflation of hydrochloric acid may not be explained by the factor of acidity alone, because the same quantity of acid may be given rabbits intravenously with impunity. Consequently, death must be ascribed to some local effect. From the fact that other acids, for example, carbolic acid, produce thrombi when directly applied to tissues, it seemed possible that coagulation of the blood within the alveolar capillaries followed the acid insufflation, and such a local coagulative action was shown to take place. The process will be described in detail in a subsequent paper.[4]

[4] Winternitz, M. C., Smith, G. H., and McNamara, F. P., *J. Exp. Med.*, 1920, xxxii, 211.

In subsequent experiments in which the concentration of acid was reduced to 0.25 per cent, sudden death was rarely observed; the majority of the animals came out of the anesthesia and later showed no untoward symptoms, unless pulmonary infection had occurred. Similar results followed the use of acids as dilute as 0.1 per cent. The gross and microscopic pictures found, even in animals of the same species, varied greatly, and it would not be useful to differentiate the effects of the two more dilute solutions mentioned. In general, the lesions caused by the weaker acids are less extensive and the likelihood of acute death is minimal.

With concentrations of acid as divergent as 1 per cent and 0.25 per cent variations in the effects are quantitative rather than qualitative. In a few instances death occurred shortly after the insufflation of the 0.25 per cent acid, possibly because of individual susceptibility. In such circumstances it is difficult to recognize any difference in the effects of a stronger and a weaker acid solution. However, when insufflated animals are killed after a period of only 15 minutes, it is found that the 1 per cent solution has excited the greater accumulation of fluid in the bronchi and trachea, and the fluid is more intensely stained with blood. In both instances the degree of edema is much the same. The hemorrhages, so prominent on the pleural surfaces and in the perivascular sheaths after the insufflation of a strong acid solution, are much less marked after the administration of a more dilute one. The picture described below is that secured with the less concentrated acid.

Animals killed at intervals after acid insufflation show a progressive localization of the changes within the lung. The less severely damaged areas lose their congested, edematous condition and assume a pale pink, delicate appearance. The more involved areas, because of their volume, their consolidation, and the dulling of their serous surfaces, contrast sharply with the less injured tissue. Within 48 hours the consolidated areas, red at first, become paler, even quite gray. They are usually wedge-shaped, most frequently involve the right lower lobe, and may extend from its lower border to the hilum. Occasionally the right lower lobe alone is involved. Frequently the process extends from the right lower to the left lower and the right middle lobes. Generally the line of demarcation between the dark red consolidated zone and the neighboring zone is not clear either with regard to consistency

or color. Upon section through such a consolidated area all structure is obliterated by the dark red color, but as the hilum is approached the architecture may become more distinct. Here alveoli distended with fluid can be seen and an occasional small hemorrhage. Later the fluid exudate becomes granular and small plugs protrude above the cut surface. At this stage the lung is less uniformly pigmented; gray patches interspersed with brownish ones and small, dark brown foci may occur.

How early this reaction takes place is difficult to determine histologically. Serum, we know, appears in the alveoli almost immediately after the insufflation, and is associated with an intense vascular congestion. In local areas also hemorrhage into the interstitial tissue and the alveoli may be prominent. The subsequent exudative changes within the lung vary with the extent and the intensity of the initial damage. Within 48 hours there appear in the lung wedge-shaped areas which show a sterile fixation of the involved tissue now devoid of nuclei. With a less marked degree of damage the alveolar walls presenting nucleated cells are distinguishable, and their lumina are filled chiefly with erythrocytes. The extreme changes will be presented in detail in a subsequent communication.[4]

The inflammatory reaction associated with milder grades of injury deserves emphasis. Within 8 hours after the damage the epithelium of the bronchioles and of the atria becomes converted into a red, ribbon-like strand devoid of nuclei. This, often thicker than the original epithelium, is raised like a blister from its basement membrane by a serofibrinous exudate in which polymorphonuclear leucocytes are found occasionally. The dilated atria and the alveolar ducts, lined with red, are conspicuous. In small areas the thickened alveolar walls show no cell nuclei. Atelectasis is not present and the degree of dilatation of the alveoli varies. The alveoli are filled with an exudate rich in serum and fibrin which contains desquamated alveolar cells. Later to this exudate are added polymorphonuclear cells which earlier were more conspicuous not only in the alveolar walls, but also in the sheaths of the bronchi and vessels. Red blood cells are also found, and, in fact, all grades of exudate occur, from those composed entirely of closely packed erythrocytes to those in which these cells are scanty or absent altogether.

The dilatation of the alveolar ducts and the hyalinosis of the bronchiolar epithelium after acid insufflation are especially noteworthy. These phenomena were characteristic both of the lesions subsequent to influenzal pneumonia and of those after exposure to corrosive gases. The terminal bronchioles are converted into conspicuous, irregular sacs, which may compress the adjacent alveoli. The marked hyalinosis, it is clear, originates from an acute necrosis of the epithelium. Nuclei can often be seen at the base of the ribbon, and in the earlier stages cell demarcation is evident. Later, when nuclei and cell boundaries disappear this membrane grows by accretion with the addition of fibrin or other material, so that ultimately it becomes thick and tends to occlude the distended bronchioles and ducts.

SUMMARY.

Acid administered to rabbits by intrabronchial insufflation causes an immediate and extreme damage of the lung tissue. Within certain limits the degree and extent of the injury vary according to the concentration of the acid. With the greater concentrations death occurs promptly, almost immediately, and the lethal process has associated with it a decreased permeability of the pulmonary vessels. The latter fact has been confirmed by postmortem arterial injections. With weaker solutions the results, grossly and histologically, resemble those noted after influenzal pneumonia and gas poisoning. Thus, there is destruction of the epithelium of the bronchioles, the alveolar ducts, and the alveoli. The extent of the damage to the alveolar walls varies. Exudation occurs into the alveolar, interstitial, perivascular, and peribronchial tissues. Primarily this exudate is serous, but a rapid deposition of fibrin occurs, and later polymorphonuclear leucocytes and erythrocytes accumulate. In different animals, or in different portions of the same lung, there may be consolidations of different types, serofibrinous, hemorrhagic, or purulent, with or without destruction of the alveolar walls. Subsequent changes relate to the organization of the exudate, necrosis, proliferation of the epithelium in the alveoli and bronchi, and, finally, to the regeneration of the pulmonary parenchyma.

Experiments now in progress indicate that similar changes can be induced by various acids, both inorganic and organic. Experiments also show that similar changes are produced in other species of animals but that species differ in their resistance according to the acid and the concentration in which it is employed.

CONCLUSIONS.

Intrabronchial insufflation of weak solutions of hydrochloric acid, from 0.1 to 0.25 per cent, in rabbits causes an inflammatory process which resembles that encountered in influenza and after the inhalation of toxic gases. There are intense edema and congestion of the lung with hemorrhage, dilatation of the alveolar ducts and bronchioles, hyaline necrosis of the epithelium of these structures, and lobular, pseudolobular, and even lobar types of consolidation, which are more frequently serofibrinous but may be hemorrhagic or even purulent.

EPITHELIAL PROLIFERATION FOLLOWING THE INTRA-BRONCHIAL INSUFFLATION OF ACID.

By M. C. WINTERNITZ, M.D., G. H. SMITH, Ph.D., and F. P. McNAMARA, M.D.

(*From the Brady Laboratory of Pathology and Bacteriology of the School of Medicine, Yale University, New Haven.*)

Plates 8 to 11.

(Received for publication, March 31, 1920.)

Hydrochloric acid insufflation into the bronchi of rabbits causes necrosis of a degree which varies with the amount and the strength of the solution employed. With extensive damage large areas of the lung undergo fixation, and hemorrhagic infarcts may develop. A more superficial injury results in a sterile pneumonia, and the repair of the pulmonary damage begins quickly. Coincident with the formation of granulation tissue and organization of the exudate, there is a proliferation of alveolar and bronchiolar epithelium. Overproduction of the epithelium occurs with the formation of polypi in the bronchioles, and even extension of the epithelium into surrounding tissue. Although invasion of preexisting healthy tissue by the proliferating epithelium has not been observed, the ultimate results of the process have not been studied. At this time only the early changes will be presented.

Epithelial Changes after Influenzal Pneumonia.

Reference has been made elsewhere[1] to the resemblance of the lesions which follow the intrapulmonary application of acid to those associated with influenzal pneumonia, and here it may be pertinent to mention the unusual epithelial changes not infrequently seen after fatal influenzal pneumonia. Alveoli lined by large, actively dividing cells are numerous, and desquamated cells only are found in the lumina. Other portions of the lung present different stages of acute reaction,

[1] Winternitz, M. C., Smith, G. H., and McNamara, F. P., *J. Exp. Med.*, 1920, xxxii, 199.

or even of organization. The epithelium of the larger air passages —trachea, bronchi, and bronchioles—shows active regeneration. In the bronchioles especially the epithelium may not only grow over a granulating exudate in the lumen, but may invade the surrounding alveoli. It is difficult to differentiate such a cellular reaction from an invasive epithelial neoplasm. Similar reactions have been observed after the intrabronchial insufflation of hydrochloric acid.

Epithelial Changes after Intrabronchial Insufflation of Hydrochloric Acid.

The experimental procedure here was identical with that employed previously, and, therefore, need not be described in detail. Rabbits were insufflated with 5 cc. of 0.25 per cent hydrochloric acid.[2] The description of the lesions consequent to such a procedure follows.

Proliferation of the Alveolar Epithelium.—This is seen as early as 36 hours after insufflation; in 52 hours many alveoli have an entirely new layer of lining cells. As a rule, these alveoli contain but little exudate. Serum, occasional fibrin strands, or erythrocytes occur, but large, pale-staining, polyhedral cells are more frequent. The alveolar space is small, attributable in part to the size of the lining cells. These large cells with granular protoplasm and vesicular nuclei often show mitoses. Occasionally a syncytial-like mass of newly formed epithelial cells is found. The karyokinetic figures appear quite regularly. Multipolar division is not encountered. Where many alveoli within a local area have a new epithelial lining, the intense blue staining of the area attracts attention, even under low magnification.

Proliferation of the Bronchiolar Epithelium.—The bronchiolar epithelium affords the best opportunity to study the reparative process. The acid, it will be recalled, kills the epithelium, which then stains intensely with eosin as a red ribbon. For some time this material adheres to the basement membrane, and, by the addition of fibrin or other substance, it may become even more conspicuous than in earlier stages. Gradually, however, it separates from the basement membrane and, folded upon itself, lies in the lumen. If the necrosis has not extended below the basement membrane and a few of the cells have been spared, these quickly begin to divide, and at the end

[2] Before insufflation the animals were completely anesthetized.

of 36 hours a portion of the bronchiolar wall is lined by young cells. These cells are conspicuous because of their size, the homogeneity of their protoplasm, the variation in the density of the nuclei, and the frequency of mitotic figures. The dead epithelium is often separated from the basement membrane by the development of these young cells (Fig. 1).

The necrosing action of the acid may extend through the epithelial lining of the bronchioles into the surrounding tissue. When this occurs the young epithelium finds an obstacle to its regeneration along the basement membrane. Where normally there should be a single layer of cells, several are found in a sector of a tubule in which the basement membrane is intact. When these young cells reach the point where necrosis involves the deeper structures, their pathway is obstructed. Consequently, they pile up and penetrate the pulmonary parenchyma (Fig. 2). As it extends, the epithelium selects the walls of surrounding necrotic alveoli for scaffolding (Fig. 3).

The ability of this young epithelium to proliferate is extraordinary. Even when the basement membrane of a tubule is intact, it may be lined by several layers of cells within 52 hours. The dead epithelium which has been replaced is found in the lumina of the bronchioles, and there may provide a surface for the extension of the new epithelium. A picture results similar to that obtained in tissue cultures. The bronchiole, relined with an epithelial coat several times as thick as normal, shows a mass of young cells, in contact with the epithelium at one point, extending over the dead membrane within the lumen. Presumably the young epithelium feeds on the necrotic material and conditions not unlike those present in tissue cultures provide for the proliferation (Fig. 4).

This source of nutriment is merely temporary, of course, and the new epithelium would die if more favorable conditions were not provided. The invasion of fibroblasts and capillaries may supply such a deficiency (Fig. 5). In Fig. 5 the stroma has penetrated the basement membrane of the tubule to afford the necessary scaffolding and vascularization of the epithelium, and the result is a typical epithelial polyp in the bronchus. This followed within 8 days after the acid insufflation, and the epithelium of the bronchus, both on the basement membrane and in the polyp, shows continued evidence of proliferation.

Proliferation Following Damage of Large Areas of Tissue.—The most striking reparative processes are those encountered where relatively large areas of tissue have been killed by the insufflated acid. Here the architecture is still discernible. The absence of polymorphonuclear reaction is remarkable; aside from some coagulated material, the nature of which is indeterminate, the lumina of alveoli and bronchi remain free. Proliferation of the granulation tissue from the surrounding healthy lung rapidly changes these areas. The epithelium is very active and grows into the dead material along the walls of the bronchioles and alveoli. Attempts to form new alveoli are noted at the periphery of such a necrotic zone. These alveoli are usually small, lined by high epithelium, and separated from each other by fresh granulation tissue. The picture resembles that of a fetal lung.

In other places epithelial activity is less uniform. Irregular clefts and cavities, lined by rapidly dividing epithelium, are found in the newly formed stroma (Fig. 6). The picture may easily be confused with that of a malignant neoplasm.

The extent of the epithelial proliferation varies in different individuals. The results described above represent the most marked changes in a relatively large series of animals studied as late as 10 days after insufflation. In every instance the proliferation, even when extensive, was regular. Direct division of cells was not observed and the mitoses were normal without evidence of variation in the chromatin content of the daughter cells. Nuclear division without corresponding division of protoplasm has been found occasionally.

CONCLUSIONS.

The damage caused by the introduction of acid into the pulmonary parenchyma is repaired rapidly.

The depth of the necrosis determines which elements will participate in the repair. If epithelium alone is injured, epithelium alone takes part in the repair. When the damage involves the deeper tissue, organization by granulation competes with and impedes the development of the epithelium.

An overproduction of the epithelium occurs and may form bronchiolar polypi or extend into the peribronchial tissue.

EXPLANATION OF PLATES.

Plate 8.

Fig. 1. 36 hours. The necrotic epithelium has separated from the basement membrane, and one sector of the bronchiole shows regenerating lining cells.

Fig. 2. 52 hours. Normal extension of the young epithelium is obstructed where the necrosing process involves the deeper tissues of the bronchiolar wall. Here the cells tend to invade the surrounding tissues.

Plate 9.

Fig. 3. 5 days. Many layers of newly formed epithelium line the small bronchiole and at one point extend into the surrounding tissue. The alveoli also are lined by new epithelium. Mitotic figures are frequent.

Plate 10.

Fig. 4. 52 hours. Where the necrotic material is in contact with the newly formed bronchiolar epithelium, it serves as a scaffolding upon which the young cells extend.

Fig. 5. 5 days. Vascular granulation tissue has invaded a mass of epithelium, as seen in Fig. 4, through the bronchiolar wall. The result is a typical polyp.

Plate 11.

Fig. 6. 8 days. The newly formed alveoli to the left have the appearance of fetal tissue. On the right the necrotic lung is being invaded by granulation tissue and rapidly developing epithelium.

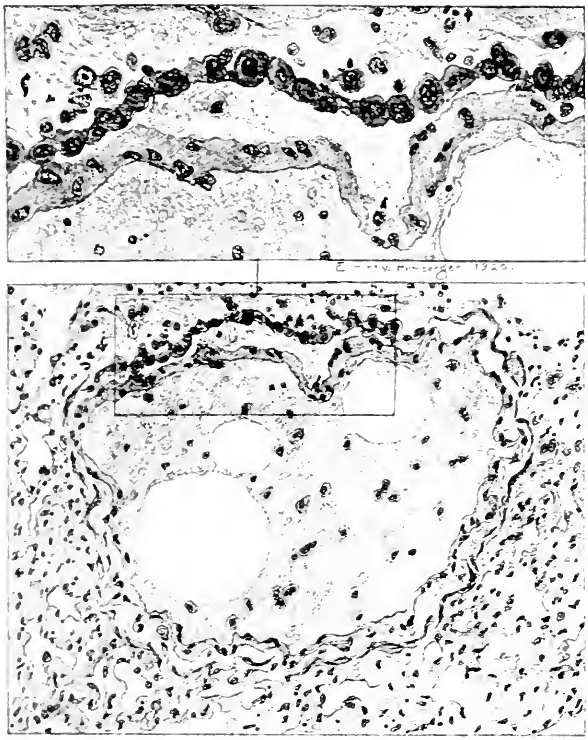


FIG. 1.

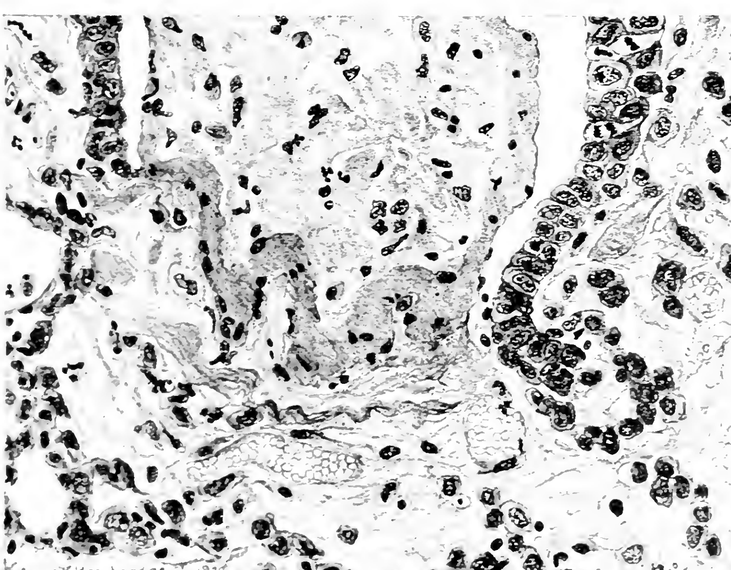


FIG. 2.

(Winternitz, Smith, and McNamara: Intrabronchial insufflation of aci .)

FIG. 3.

(Winternitz, Smith, and McNamara: Intrabronchial insufflation of acid.)

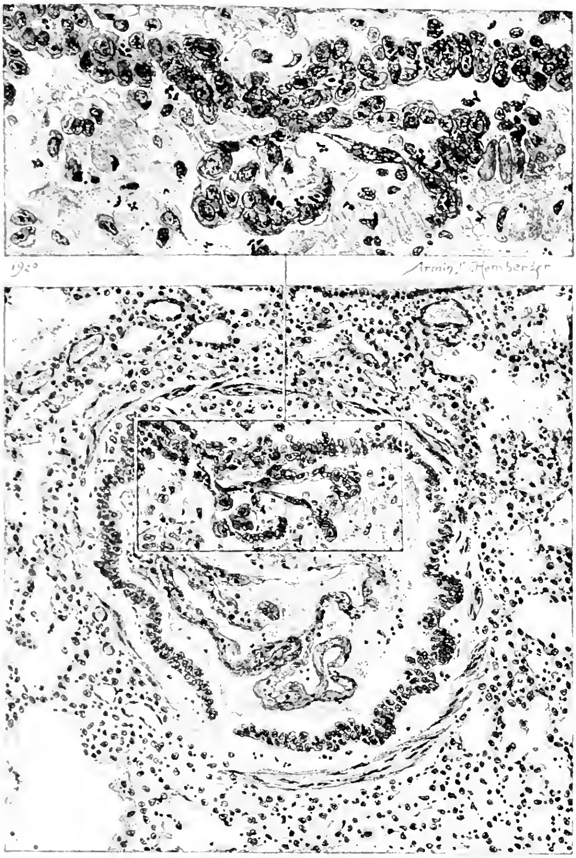


FIG. 4.

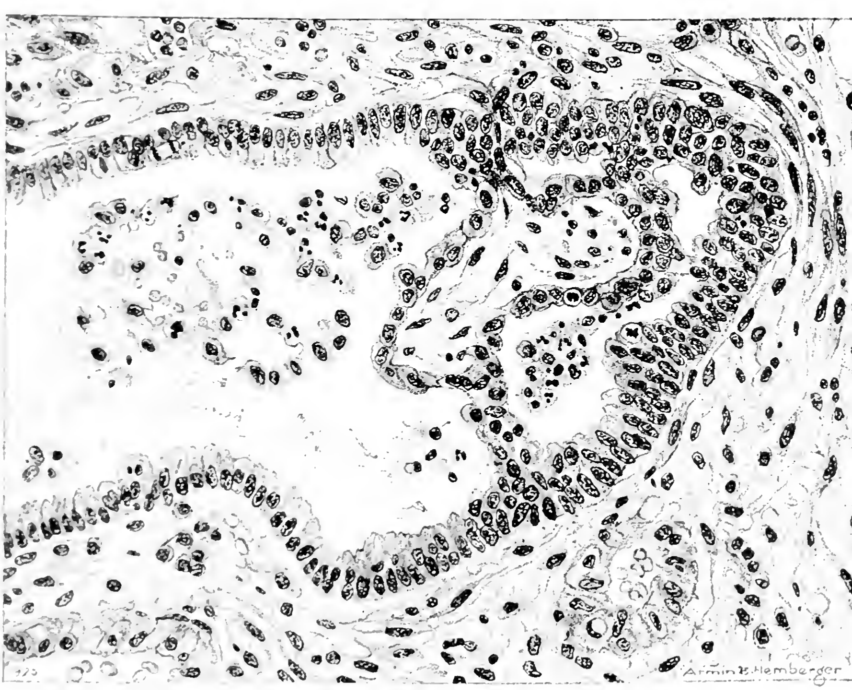


FIG. 5.

(Winternitz, Smith, and McNamara: Intrabronchial insufflation of acid.)

Fig. 6.

(Winternitz, Smith, and McNamara: Intrabronchial insufflation of acid.)

PRODUCTION OF PULMONARY INFARCTS BY THE INSUFFLATION OF ACID.

By M. C. WINTERNITZ, M.D., G. H. SMITH, Ph.D., and F. P. McNAMARA, M.D.

(*From the Brady Laboratory of Pathology and Bacteriology of the School of Medicine, Yale University, New Haven.*)

Plates 12 and 13.

(Received for publication, March 31, 1920.)

Infarcts of the lung, both in man and in experimental animals, result either from infected emboli in a normal organ or from bland emboli when cardiac decompensation or other disturbance has decreased the efficiency of the pulmonary circulation.[1] Such an explanation appeared adequate, since hitherto the infarcts so commonly found have been associated with the occlusion of a large supplying vessel with secondary extension of the thrombus into the smaller branches. A reverse of this process is shown in the experiments recorded below. The insufflation of acid into a normal lung leads to infarction, the primary process being a coagulative change in the capillary bed with secondary propagation of the clot into the larger vessels.

Extensive vascular injury in the pulmonary tissue occurs after the inhalation of irritating gases and also in influenzal pneumonia. Clinically, this is attested by the presence of blood in the sputum, and in both these conditions at autopsy hemorrhage into the lung is not infrequent. Following the inhalation of irritating gases, the pulmonary hemorrhages are often confined to the perivascular sheaths, but may involve the pleuræ, alveoli, and bronchi. These hemorrhages are rarely circumscribed in such a way as to be confounded with infarcts. In influenza the hemorrhage may be diffuse and not unlike that encountered after the inhalation of irritating gases; on the other hand, the red blood cell extravasation may be localized and definitely

[1] Karsner, H. T., and Ash, E. J., *J. Med. Research*, 1912–13, xxvii, 205.

circumscribed.[2] Indeed, the literature on the pathology of influenza refers to lesions, which, from their description, cannot be differentiated from infarcts. However, they have not been designated as infarcts, although the term "infarct-like areas" has been applied to them. Uncertainty with regard to this lesion is probably referable to the conception that bland infarcts occur only in a lung the circulation of which is impaired by cardiac decompensation or by compression of the pulmonary tissue by fluid or other substance foreign to the pleural cavity. A further difficulty in identifying these hemorrhagic consolidated areas as infarcts may have arisen through the fact that, although thrombi are frequent in the lung after pulmonary irritating gases and in influenzal pneumonia, a relation between them and infarcts has not been demonstrated. Many thrombi are unassociated with such foci, and on casual inspection at least, it is not always possible to demonstrate antemortem occlusion of the larger vessels leading to such infarct-like zones. Consequently, no explanation for the production of these infarct-like areas has been forthcoming, in spite of the fact that vascular lesions (thrombi) involving the smaller vessels in the alveolar walls and even the larger arterioles have been described by many observers.

In a previous communication[3] the results of intrapulmonary insufflation with an inorganic acid (hydrochloric) have been reported. Here it may be reiterated that the extent of the damage to the lung varies with the strength of the acid solution employed. When a 1 per cent solution of hydrochloric acid is insufflated into the lung of a rabbit,[4] the animal succumbs acutely, and during the few minutes it remains alive after the treatment, blood-stained fluid froths from the mouth. With weaker solutions the animals do not die acutely, and the destruction of the lung with subsequent inflammatory exudation is more outspoken.

[2] Winternitz, M. C., McNamara, F. P., and Wason, I. M., Pathology of influenza, New Haven, 1920.

[3] Winternitz, M. C., Smith, G. H., and McNamara, F. P., *J. Exp, Med.*, 1920, xxxii, 199.

[4] Before insufflation the animals were completely anesthetized.

Lesions in Animals That Survived Only 15 Minutes.

A few of the animals insufflated with 5 cc. of 0.25 per cent hydrochloric acid died within 15 minutes. In all of them the gross picture is identical and is illustrated in Fig. 1. The voluminous lungs are slightly congested throughout, but the most constant and marked changes occur in the lower lobes, usually the right. The increase in size is much more marked in the affected lobe than elsewhere. The lower third, and sometimes even half of the lobe, is garnet, and the color is accentuated by the glistening, smooth pleural surface through which it is seen. The discolored area of the lung is non-air-containing. On section, as on the surface, the line of demarcation is sharp and defines a wedge-shaped zone whose apex approaches the hilum (Fig. 2). Histologically, sections through such a consolidated area show a mass of well preserved erythrocytes filling the alveoli and even the bronchi. The alveolar and bronchiolar walls may still be made out; though shrunken, their cellular elements are intact and the nuclei stain (Fig. 3).

Lesions in Animals Killed at Varying Intervals after Insufflation of Acid.

Animals which survived the insufflation of acid in this concentration were killed at intervals up to 2 weeks after the operation, and examination of the lungs of such a series indicates clearly that there is a variation in the effects of the acid in animals of the same species. At times there are no areas of consolidation and discoloration like those described for the previous group, and the extent of such change varies from small foci to areas which involve the greater portion of a lower lobe. Especially in their lower half, these larger infarct-like areas often present a dull, even pale brown, surface. Above this zone, either sharply or gradually, the color becomes almost black. The surface markings are an index to the color change within the consolidated portion of the lung, and microscopic examination of the paler areas offers the most suggestive explanation of the etiology of the change. Sections from the dark, almost black portion of the consolidated lung are similar to those just described (Fig. 3). The alveoli are packed with disintegrating red blood cells, while the alveolar and bronchiolar walls are still well defined, and the cell nuclei retain

their affinity for hematoxylin. Evidence of organization and epithelial regeneration appears about the area of hemorrhagic extravasation, but discussion of this phenomenon will be deferred. The hemorrhage in the alveoli becomes less marked in sections from the paler areas. Here, the alveoli and bronchi contain a homogeneous, eosin-staining material; few red blood cells are included in the exudate. The most interesting changes occur in the alveolar and bronchiolar walls, which are swollen and often measure several times their normal width (Fig. 4). The nuclei do not stain with hematoxylin, and in the portions most affected no structure can be made out in the walls.

Striking infarct-like lesions have been produced in dogs also, by the insufflation of acid (20 cc. of 1 per cent hydrochloric acid). In this species the large size of the pulmonary vessels makes it easier to study those leading to the area of consolidation, and multiple sections frequently show them distended and occluded by a firm, red clot (Fig. 2). Histologically, in all animals, including rabbits and dogs, thrombosed vessels are found. The clot has the appearance of a propagated thrombus. It is devoid of the typical thrombotic architecture, and consists largely of red blood cells with leucocytes scattered more or less regularly among them. Occasional foci of platelets with radiating fibrin strands are present.

Explanation of Infarct-Like Areas.

The explanation for the unusual production of infarct-like areas after the insufflation of acid is not evident from the histological study of the more typical lesions. However, as has been said above, the microscopic picture of the paler brown peripheral zone that occurs when rabbits survive for several days, offers a clue toward the interpretation of the process. Here the swollen alveolar walls are necrotic (Fig. 4). The alveoli may contain serum and fibrin, but no cells—an unusual picture in the lung. The process resembles gangrene more than any other familiar lesion of this organ, and bears a strong resemblance to an anemic infarct in the spleen or kidney. The walls of the alveoli stain homogeneously with eosin; the architecture is obscured, but now and then either the lining cells or the vessel within the wall can be distinguished. Similar changes in the alveolar walls

have been described in influenza, and they also develop rapidly after the inhalation of irritating gases. It is impossible to distinguish the vessels, and frequently the blood and other constituents of the wall are so intimately fused that the individual elements can no longer be differentiated. The picture is not unlike that resulting from the application of carbolic acid to the gastric mucosa. In all probability the insufflated acid causes a fixation of the tissues with simultaneous inhibition of all cell activities. Consequently, the intermediary regressive processes dependent upon the loss of balance of the cellular ferments are lacking.

This picture results, as has been said, when the more concentrated acid is employed, and, therefore, may be considered to represent a maximal effect. Milder grades of damage also may result in thrombosis of the small vessels. If vessels in sufficient number are occluded, the resistance to the flow of fluid in the lung should be demonstrable by pulmonary arterial injection, and to test this point a series of experiments was devised.

It was found that a longer period of time than in normal subjects was required for a Berlin blue gelatin injection mass to appear on the venous side, even in animals that had succumbed within a few minutes after the insufflation of a 1 per cent solution of hydrochloric acid. The lung cannot be injected uniformly; and microscopically, in thick frozen sections, it is readily seen that many vessels are not permeated by the mass. When local, consolidated, red areas have developed, increased pressure, even after the dye has appeared on the venous side, does not result in injection of the consolidated zone. This zone contrasts sharply with the neighboring area, deeply stained by the dye in its vessels.

The injection method thus provides the means of determining the effects of the acid more minutely. The lung may appear uniformly blue, while, in fact, numerous, small areas, scattered through one or more lobes, contain none of the gelatin. On the other hand, a large area may be completely uninjected and sharply demarcated. Between these extremes there are many variations. Histological examination indicates that the acid coagulates the blood in the swollen vessels, and the phenomenon is actually demonstrated upon injection. By this method variations in the gross picture are explained through localization of the coagulative process.

Our immediate interest lies in the instances in which the action of the acid is confined to a local area. Where this occurs it is possible to show that large vessels leading to the sharply demarcated zone are distended with clot. However, these clots are not found in the animals that die within a few minutes; they are most marked after a period of several days.

From the above experiments we conclude that when concentrated acid reaches the alveolar walls, necrosis of the entire structure and thrombosis of the vessels follow. The resulting lesion depends on the extent of the necrosis and thrombosis. When a large number of vessels becomes occluded in a given area, the possibility of the propagation of the clot into the larger vessels is increased, and when this occurs, the lesion cannot be differentiated from an infarct.

Infarction in tissues with an extensive collateral circulation does not occur except under extraordinary circumstances. Therefore, one would hardly expect this process in the liver, and no really authoritative cases of infarction of the liver have been reported. Nevertheless, a liver lesion which can easily be confused with infarction has been observed recently in two cases in which the capsule of the organ was torn and the rent extended several centimeters into the parenchyma. Both individuals lived several days after they were injured. At autopsy, in each case, the hepatic tissue surrounding the clot at the point of rupture was the seat of a coagulative necrosis indistinguishable from the type of necrosis which occurs in infarcts of the spleen and kidney. When the liver was torn the smaller capillaries within the lobules were broken, and with the cessation of hemorrhage, clots formed at the mouths of these capillaries. These clots were propagated into the radicles of both the hepatic artery and portal vein, so that the extensive collateral circulation within the lobule, the safeguard against all but extraordinary conditions, was rendered ineffective. It would seem that this is a type of infarction that can result from the plugging of peripheral vessels with subsequent propagated clots into the larger supplying vascular bed. Unquestionably, this is the process by which infarcts are formed after insufflation with acid; and, in all probability, the infarct-like areas associated with influenza also result from similar damage to the intraalveolar capillaries or arterioles which undergo thrombosis.

CONCLUSIONS.

Intrabronchial insufflation of acid causes immediate necrosis of the walls of many alveoli. Thrombosis of the alveolar vessels is an associated phenomenon. When a large number of vessels becomes affected, a clot propagates rapidly into the larger supplying vessels. The resulting lesion is indistinguishable from a hemorrhagic infarct.

The infarct-like areas so frequently encountered in influenzal pneumonia, it is not unlikely, have their origin in a similar process.

Infarction depends not only upon thrombosis or embolism of the large vessels, but may be initiated by extensive damage to the capillary bed. By this process infarcts may form in organs which are normally protected by collateral circulation.

EXPLANATION OF PLATES.

Plate 12.

Fig. 1. Infarct of the lung produced by the insufflation of 5 cc. of 0.25 per cent hydrochloric acid.

Fig. 2. Cross-section of an infarct of the lung. The larger supplying artery is occluded by a propagated clot.

Plate 13.

Fig. 3. Histology of an infarcted area. The alveolar walls are thin and the alveoli are distended with erythrocytes.

Fig. 4. Section showing the maximal effect of acid on the lung tissue. The alveolar walls are necrotic and their vessels contain hyaline thrombi.

218.

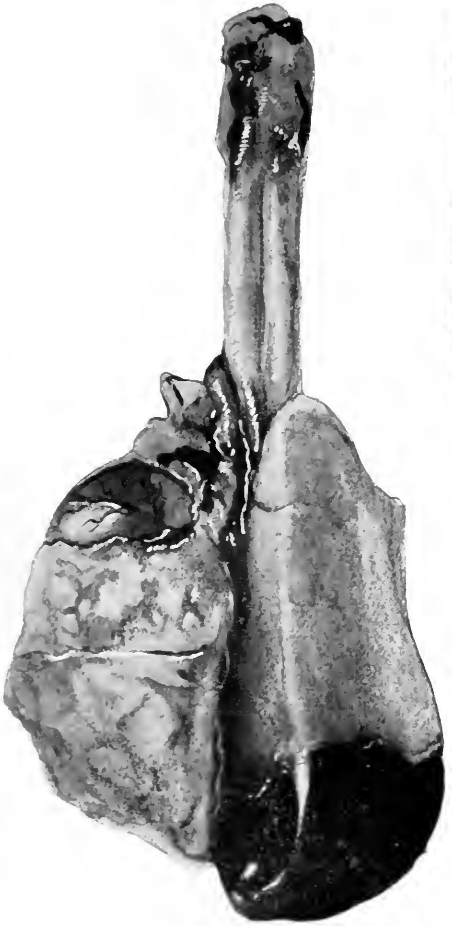


FIG. 1.

FIG. 2.

(Winternitz, Smith, and McNamara: Intrabronchial insufflation of acid.)

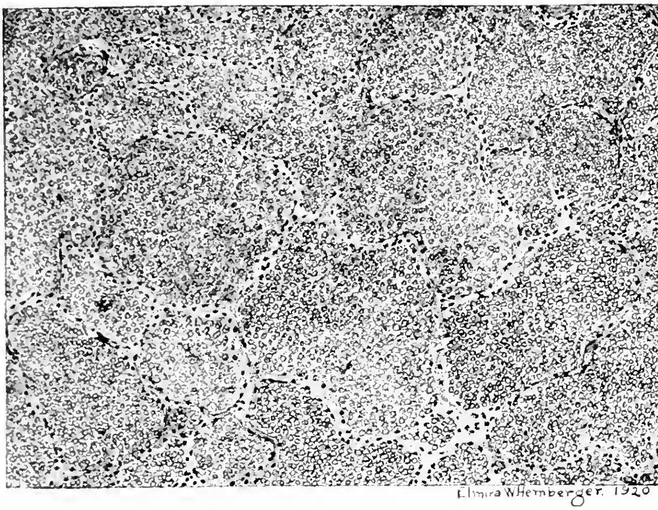


FIG. 3.

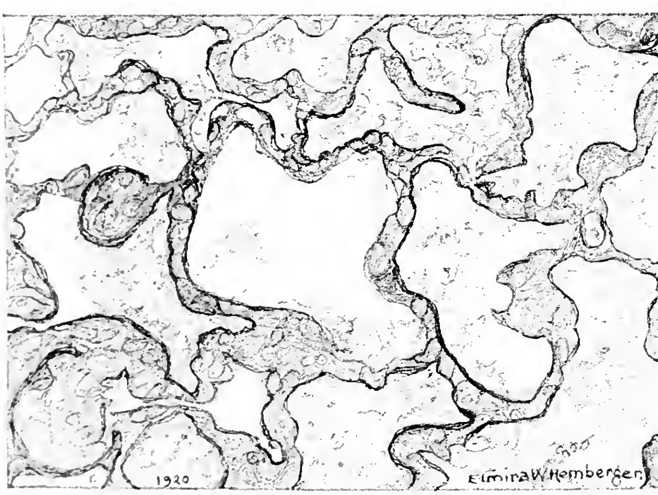


FIG. 4.

(Winternitz, Smith, and McNamara: Intrabronchial insufflation of acid.)

A STUDY OF BACILLUS PYOGENES.

By J. HOWARD BROWN, Ph.D., and MARION L. ORCUTT.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

Plates 14 to 16.

(Received for publication, April 20, 1920.)

INTRODUCTION.

Bacillus pyogenes is associated with various disease processes of swine and cattle and is not infrequently found in milk. Failure to recognize it as the *B. pyogenes* described in French, Dutch, and German literature may be due to certain difficulties in its cultivation, to its close resemblance to streptococci under certain conditions, and to the fact that it is usually found in mixed culture with organisms which may mask its presence.

In France, according to Lucet (1893), next to the streptococci *B. liquefaciens pyogenes* is one of the most frequently found organisms in suppurations of cattle. In Germany Künnemann (1903) found a similar organism, which he calls *B. pyogenes bovis,* in 90 per cent of suppurations of cattle. Grips (1898) found *B. pyogenes suis* commonly present in pleuritis and peritonitis of swine. Tuff (1906) found *B. pyogenes* in over 13 per cent of milk samples examined. Glage (1902–03) found the organism third in importance to streptococci and *B. tuberculosis* as a cause of mastitis in cows. Eggink is quoted by Ward (1917, *a*) as having found *B. pyogenes* of first importance in metritis of cattle. There are no statistics as to the prevalence or distribution of the organism in America. Ward (1917, *a*) found it frequently in swine and cattle.

Lucet (1893) studied 52 cases of suppuration, cold abscesses, traumatic abscesses, and cases of septicemia, all in cows. He says: It seems that there exist in the cow special pyogenic microbes, not yet described, which are a streptococcus, a staphylococcus, and three bacilli. He names these organisms *Streptococcus pyogenes bovis*, *Staphylococcus pyogenes bovis*, *B. pyogenes bovis*, *B. liquefaciens pyogenes bovis,* and *B. crassus pyogenes bovis.* His description of *B. liquefaciens pyogenes bovis* agrees with that of the *B. pyogenes* of Grips, Glage, Künnemann, and others. Lucet gives a photograph of the organism and describes it as non-motile, liquefying gelatin slowly, not growing on potato, growing as a sediment in veal bouillon without producing turbidity, non-virulent for guinea pigs, injected into rabbits intravenously producing subaponeurotic abscesses principally in the limbs where they sometimes acquire great size but do not discharge. His *B. pyogenes bovis*

resembled the above morphologically but did not liquefy gelatin and its pathogenicity for guinea pigs was variable. *B. crassus pyogenes bovis* was a larger motile bacillus. Grips (1898) described *B. pyogenes suis* and the lesions of the pleura and peritoneum from which it was isolated. Poels (1899) described a similar organism from polyarthritis of calves and called it the polyarthritis bacillus. Künnemann (1903) described *B. pyogenes bovis* from suppurations of cattle. Glage (1902–03) made a careful comparison of *B. pyogenes suis* (Grips) and *B. pyogenes bovis* (Künnemann) and concluded that they were identical. He proposed that the organism be called *B. pyogenes*. His contention that the organisms from swine and cattle are identical has not been seriously questioned. Careful bacteriological studies have been made by Koske (1906), Berger (1908), and Holth (1908). Ward (1917, *a* and *b*) has given valuable brief summaries in English, and Glage (1913) in German.

Source of Strains.

The strains of *Bacillus pyogenes* of bovine origin employed in this study were received from Dr. Theobald Smith who supplies the following data concerning their source.

Strain I. From pneumonic lungs of a calf. Killed when 33 days old.[1]

Strain II. From pneumonic lungs of a calf. Killed when about 5 weeks old.[1]

Strain III. From pneumonic lungs of a calf. Killed when 38 days old.[1]

Strain IV. From pus filling both horns and body of uterus of a cow. Uterus obtained after slaughter of cow.

Strain V. From a similar case as that of Strain IV. Uterus contained a foul smelling fluid.

Strain VI. From purulent contents of uterus in case of prolapse of vagina and external os. Uterus obtained when cow was slaughtered.

Strain VII. From pneumonic lungs of a calf. Killed when 31 days old.

Strain VIII. From the uterine contents and ovaries of a case of purulent metritis and of central necrosis and pus formation in both ovaries. Associated with *B. actinoides*.

Strain IX. From chocolate-colored, offensive fluid contained in uterus of a cow slaughtered. Other bacteria present.

Strain X. From kidney, liver, and lungs of the fetus of Cow 259. Several other species of bacteria present in small numbers.[2]

Strain XI. From the fourth stomach, liver, and lungs of the fetus of Cow 291.[2]

Strain XII. From the liver of the fetus of Cow 339. Pure culture of *Vibrio fetus* isolated from the lungs.

---

[1] It is highly probable that in these cases *Bacillus pyogenes* was secondary to *Bacillus actinoides* (Smith, T., *J. Exp. Med.*, 1918, xxviii, 333).

[2] Smith, T., *J. Exp. Med.*, 1919, xxx, 325.

Strain S 1 was isolated by Dr. Carl TenBroeck from the pneumonic lung of a case of hog-cholera. Other organisms were also present in the lung.

Cultural Study.

The known morphological and cultural characteristics of *B. pyogenes* as described by Lucet (1893), Grips (1898), Poels (1899), Künnemann (1903), Glage (1902–03), Koske (1906), Berger (1908), and Holth (1908) have been summarized by Glage (1913), Buchanan and Murray (1916), and Ward (1917, *b*). They are briefly as follows:

The organism is a small slender rod 0.2 to 3.0 microns in length by 0.2 to 0.3 microns in thickness. It is quite pleomorphic being often coccoid, club-shaped, or slightly curved. It is non-motile and produces no spores. Some authors (Glage, Künnemann) have regarded it as Gram-negative and others as Gram-positive (Berger, Holth, Olt, Ward). Berger found it Gram-positive if subjected to sufficient exposure to the iodine solution. Capsules are not produced.

It is stated by most authors that the organism grows very poorly or not at all in standard bouillon or on standard agar, and that it requires hemoglobin, blood, or serum in the medium. Good growth occurs on blood or serum agar. Coagulated blood serum is slowly liquefied beginning in about 48 hours as small depressions underlying each colony. Because of this characteristic this medium has been a favorite one for isolating the organism. Milk is coagulated in about 48 hours and the curd is subsequently slowly dissolved or digested. In liquid serum or serum bouillon growth occurs in the form of a sediment. Growth does not occur at temperatures below 24°C. but in a specially prepared nutrient gelatin of high melting point Poels found liquefaction produced by the organism growing at 26°C. Growth occurs under aerobic and anaerobic conditions. Gas is not produced in carbohydrate media. According to Pütz (1904) acid is produced. Koske (1906) reports acid production in serum litmus whey. Berger (1908) obtained no growth in serum litmus whey and does not mention acid production in lactose or dextrose bouillon but notes that milk is coagulated and soured. Holth (1908) reports acid production from dextrose, fructose, galactose, maltose, lactose, and saccharose in a special meat extract (Cibil's) bouillon but obtained no acid or visible growth in the same medium containing xylose, rhamnose, arabinose, sorbose, mannitol, sorbitol, dulcitol, or glycerol. Indole, hydrogen sulfide, and nitrites are not produced. Methylene blue, litmus, and neutral red are not reduced. The bacillus is soon killed at 57°C. and is very sensitive to antiseptics.

Our experience with the organism agrees with the above as regards morphology, oxygen requirements, growth on coagulated serum, in milk, and in serum bouillon. In plain standard veal infusion bouillon made with Fairchild's peptone, however, we have obtained fairly

good growth, at least with strains which have been in cultivation a very short time. For a while the bouillon was not clouded by the culture but after cultivation for several months bouillon was distinctly clouded in 24 hours by most strains.

The production of hemolysis in blood agar by *Bacillus pyogenes* appears not to have been noted heretofore. In standard veal infusion agar plus 5 to 10 per cent of defibrinated horse blood there appear after incubation for 20 to 24 hours very small zones of hemolysis about very minute deep colonies. The colonies are often visible only under the low power of the microscope. In 48 hours the deep colonies are still quite small biconvex discs about 0.3 mm. in greater diameter but are easily seen macroscopically. The hemolyzed zones are clear, well defined, colorless, and of the beta type (Smith and Brown, 1914–15; Brown, 1919), about 1.5 to 2 mm. in diameter (Fig. 1). Isolated surface colonies do not appear so readily on the plate under aerobic conditions and the zone of hemolysis may be hardly visible. If the plate is sealed, however, individual surface colonies grow more readily and produce zones of hemolysis similar to those of deep colonies. If the blood agar plate is streaked so that many small surface colonies appear in the line of the streak, hemolysis appears beneath the streak. The individual surface colonies are very small convex colorless droplets much like those of *Bacillus influenzæ*.

We have sought to determine whether *Bacillus pyogenes* is hemoglobinophilic or whether it may be dependent upon other substances in blood for growth.

Freshly drawn horse blood was allowed to clot and a clear straw-colored serum was obtained as nearly free from hemoglobin as possible. This was used in serum agar plates.

Another portion of the same blood was defibrinated, and the corpuscles were washed repeatedly with sterile physiological salt solution. Some of the washed corpuscles were used in washed corpuscle agar plates.

Some of the washed corpuscles were laked with sterile distilled water and the corpuscle stroma removed by centrifugation. Care was taken to centrifuge the laked blood corpuscles until the supernatant hemoglobin solution no longer gave a clouding reaction with salt (Brown, 1919[3]). The hemoglobin solution so obtained was used in hemoglobin agar plates.

---

[3] Brown (1919), p. 67.

The corpuscle stroma obtained by centrifugation of the laked corpuscles was washed repeatedly in sterile distilled water and in salt solution until no visible trace of hemoglobin remained. The stroma suspension was used in stroma agar plates.

Each strain of *Bacillus pyogenes* was inoculated into the depths and streaked onto the surface of plates of the following media: (1) blood agar; (2) serum agar; (3) washed corpuscle agar; (4) hemoglobin agar; (5) stroma agar; (6) plain agar. Every precaution was taken to insure uniformity of conditions, such as use of the same lot of agar throughout, inoculation of one plate after another in the same manner and with the same amount of material. The plates were inoculated from fresh plain bouillon cultures. Observations were made as to the amount of growth, size and number of colonies, and morphology of the organisms within the colonies. In every case the best growth was obtained in blood agar. Next best was the growth in washed corpuscle agar and in serum agar. In the majority of cases better growth was obtained in stroma agar than in hemoglobin agar. Little or no growth occurred in plain agar. The results indicate that serum is of as much importance as corpuscles, and that hemoglobin is probably the least essential of the blood constituents for the growth of this organism. That hemoglobin does not satisfy the requirements of *Bacillus pyogenes* as it does those of *Bacillus influenzæ* is indicated by the following experiment. A blood agar plate was inoculated in the depths and also streaked with *Bacillus pyogenes*. At a point near the streak a large zone of laking was produced by depositing a bit of saponin on the surface of the medium. Both deep and surface colonies within the zone were no larger and no more numerous than elsewhere in the plate (Fig. 1). Under similar conditions colonies of *Bacillus influenzæ* grew more luxuriantly in and near the zone of laking than elsewhere in the plate. A similar result was obtained by producing zones of laking by directing a stream of carbon dioxide against the bottom of the plate until a spot was frozen. The blood used for such experiments must be fresh; otherwise there will be sufficient free hemoglobin in the serum to obliterate the difference in growth between that in the laked zone and elsewhere.

The fact that colonies of *Bacillus influenzæ* grow more luxuriantly in the vicinity of colonies of hemolytic streptococci and staphylococci has

been known for many years (Grassberger, 1897). According to Davis (1917) there is involved in this phenomenon not only hemoglobin but also a vitamine which is supplied by the foreign organism or may be supplied by fresh sterile vegetable or animal tissues. We have not found the growth of *Bacillus pyogenes* colonies to be augmented by proximity to colonies of other organisms.

That blood is not absolutely necessary for the growth of *Bacillus pyogenes* is shown by the fact that it grows very well on Dorset's egg medium. On this medium the colonies, especially those on the upper half of the slant, produce little pits like those produced on coagulated serum. The organism also grows fairly well on a medium consisting of three parts of white of egg plus one part of standard veal infusion bouillon, slanted and coagulated in the inspissator. On this medium the streak of growth also produces some depression as on coagulated serum. Even an old laboratory strain of *Bacillus influenzæ* which has become quite easy to cultivate grows very slightly on Dorset's medium and not at all on the egg white medium.

Staining and Morphology.

Bacillus pyogenes stains well with dilute carbolfuchsin or with Löffler's methylene blue but we have obtained the best results with the Gram stain. Under the influence of prolonged decolorization with alcohol the organism may not retain the violet stain so tenaciously as do staphylococci, but when stained on the same slide with *Bacillus influenzæ*, *Bacillus coli*, the meningococcus, or other recognized Gram-negative organisms there can be no doubt as to *Bacillus pyogenes* being clearly Gram-positive. The disagreement in the literature as to the Gram staining of the organism may be due to the many methods in use for applying and making up this stain and the poor keeping qualities of some of them. It is also pointed out by Olt (1908) that the dead organisms found in old exudates are Gram-negative whereas the living organisms are Gram-positive. We have used Stirling's aniline gentian violet, but 1 or 2 per cent of the dye by weight rather than 5 per cent has been found sufficient. Exposure of films to the violet stain for 10 seconds, Lugol's solution for 15 to 30 seconds, decolorization in alcohol until no more color appears to be given off,

and counterstaining for 10 seconds in aqueous safranine has always given good results.

The various media employed have afforded opportunity to observe the pleomorphism of *Bacillus pyogenes*. Four or five more or less distinct forms and their intermediate stages are recognizable.

1. Bacillary Form.—These are small, short, homogeneously stained Gram-positive bacilli occurring singly (Fig. 2).

2. Fusiform Form.—In this form there are one or two strongly Gram-positive central granules while the ends of the bacilli fade out and take the counterstain to some extent. These are often slightly curved, and superficially resemble *Bacillus acne* though smaller (Figs. 3 and 4).

3. Diphtheroid Form.—These are bacilli of irregular length and contour, some clubbed, containing deeply stained bands or granules irregularly placed (Fig. 6).

4. Streptococcoid Form.—These appear exactly as Gram-positive diplococci and streptococci in small clumps and short crooked chains. If, as may be supposed, these cocci are but the granules of the diphtheroid form which have assumed a very regular size, form, and arrangement, the matrix forming the remainder of the bacillus is invisible to the eye though the photographs reveal a faint matrix which probably retained a trace of the red counterstain (Figs. 7 and 8).

5. Filamentous and Branching Form.—The bacilli appear drawn out with irregular diameter. There are few definite deeply staining granules but in some filaments there may be a deep violet portion blending gradually with a faintly stained or Gram-negative portion. Definite buds and branches were occasionally found, especially in Strain IV (Figs. 5 and 6).

Cultures often show a mixture of the above forms. Some strains have more of a tendency to assume one form and others another, but all the strains studied have produced all the various forms at one time or another. The streptococcoid form may be so definite and so habitual with certain strains that the organism may be mistaken for a streptococcus. We have spent some weeks studying what was thought to be a very unusual minute hemolytic streptococcus only to discover that we were working with *Bacillus pyogenes*.

In observing the morphology of the organism on various media we hoped to discover the factors which determined the variations in form, but the hope was only partially realized. In the lesions of animals the organism appears in the bacillary form with a tendency to be more granular in older lesions. In bouillon and serum bouillon the bacillary form predominates though any or all of the other forms may also be present; the streptococcoid form is least likely to appear. The latter form was encountered most commonly on washed corpuscle agar, stroma agar, and especially on hemoglobin agar. On blood agar there was a mixture of bacillary, granular, and streptococcoid forms. The growth on serum agar was bacillary. Long filamentous and branching forms were obtained in serum bouillon and in the condensation fluid of coagulated blood serum. Fusiform bacilli appeared most commonly in milk and in bouillon containing fermentable sugar. Our study has produced the impression that the filamentous and streptococcoid forms represent the two extremes of pleomorphism. The strains which produced filamentous forms most readily, *e.g.* Strain IV, produced streptococcoid forms with difficulty, while other strains, *e.g.* Strain X, produced streptococcoid forms readily but filamentous forms rarely. The forms most commonly encountered are the bacillary and fusiform.

Fermentation.

In fermented bouillon plus 10 per cent of sterile horse serum and 1 per cent of the test substance all the strains of *Bacillus pyogenes* produced acid from dextrose, saccharose, lactose, and xylose but not from raffinose, inulin, mannitol, and salicin. Holth (1908) reports that galactose, fructose, and maltose are also fermented. We differ from him in regard to xylose. Our fermented bouillon containing the test substances was probably more favorable for the growth of the organism than was his meat extract bouillon.

Fermentation tubes were employed for the tests and the contents of both the open bulb and the closed arm were titrated for acid after incubation of the cultures for 7 days. The results recorded in Table I show that the acidity of the bulb was much higher than that of the closed arm, little or no acid often being produced in the latter. *Bacillus pyogenes* is completely agglutinated by normal horse serum in

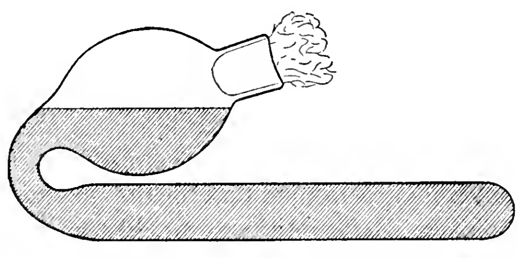
dilution of 1:100. In dextrose serum bouillon the arm of the fermentation tube remains clear and most of the growth is in the form of a sediment in the neck of the tube. If, however, the fermentation tube be incubated in the horizontal position (Text-fig. 1) an abundance of

TABLE I.

Fermentation Reactions.

Strain No.	Xylose.		Dextrose.		Lactose.		Saccharose.		Raffinose.		Inulin.		Mannitol.		Salicin.	
	Bulb.	Arm.	Bulb.	Arm.	Bulb.	Arm.	Bulb.	Arm.	Bulb.	Arm.	Bulb.	Arm.	Bulb.	Arm.	Bulb.	Arm.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
I	3.85	2.15	3.35	1.4	4.25	2.45	1.95	0.8	0.9	0.4	1.1	0.75	1.5	0.55	1.1	0.9
II	4.1	1.9	3.55	2.1	5.35	2.1	3.25	1.9	0.75	0.35	0.9	0.5	0.65	0.75	0.7	0.7
III	3.8	2.05	3.35	1.8	5.5	3.05	2.3	1.45	1.0	0.65	0.6	0.6	1.0	0.65	0.7	0.6
IV	4.0	1.9	3.9	1.2	3.35	0.85	2.75	0.9	0.85	0.4	0.75	0.55	1.0	0.65	0.75	0.4
V	3.55	2.2	3.7	1.95	3.9	1.65	2.8	1.6	1.15	0.6	0.9	0.4	0.75	0.5	0.7	0.4
VI	4.8	2.55	3.4	2.1	2.0	1.6	2.65	1.05	0.95	0.55	1.05	0.7	0.8	0.4	0.65	0.65
VII	3.35	2.55	2.6	2.2	4.05	2.35	3.75	1.65	1.2	1.0	1.3	1.05	1.15	0.08	1.6	0.85
VIII	4.4	3.2	4.0	1.5	4.6	2.5	3.2	2.7	0.65	0.45	1.0	0.9	0.95	0.5	1.1	0.65
IX	2.9	1.2	2.65	1.45	1.85	1.1	2.85	1.85	0.5	0.4	0.8	0.4	0.4	0.5	0.75	0.45
X	3.1	0.7	3.4	0.85	3.9	1.4	3.25	1.35	0.85	0.5	0.85	0.35	0.6	1.2	0.65	0.5
XI	3.75	1.8	3.85	1.5	2.8	1.05	3.4	1.15	1.1	0.8	1.2	0.4	0.75	0.45	0.8	0.6
XII	3.35	2.35	3.0	1.35	1.8	1.2	3.5	1.3	1.0	0.65	0.9	0.55	0.75	0.7	0.9	0.65
S 1	3.75	1.35	4.15	1.4	4.1	1.25	1.8	0.6	1.45	1.0	1.2	0.6	0.6	0.4	1.0	0.9

The figures indicate per cent normal total titratable acid.



TEXT-FIG. 1. Fermentation tube placed in the horizontal position.

acid is formed in the arm as well as in the bulb. These facts suggested for the moment that failure to ferment in the arm might be due to the mechanical effect of agglutination. It was discovered, however, that in plain dextrose bouillon without serum the arm was well clouded by

growth but little or no acid was produced in it. The effect of difference in oxygen tension was next considered. The closed arm of a fermentation tube of sterile bouillon containing 1 per cent of a 1:1,000 aqueous solution of methylene blue reduced by autoclaving remains colorless for weeks if incubated in a vertical position. The arm of a similar tube incubated in the horizontal position is well colored in a few hours. In the following experiment tubes of dextrose bouillon covered by 5 cc. of vaseline were placed in a boiling water bath for 30 minutes at the end of which time the methylene blue in the control tube was colorless. The other sealed tube was cooled and inoculated by means of a capillary pipette through the layer of vaseline. There was good growth in both inoculated tubes.

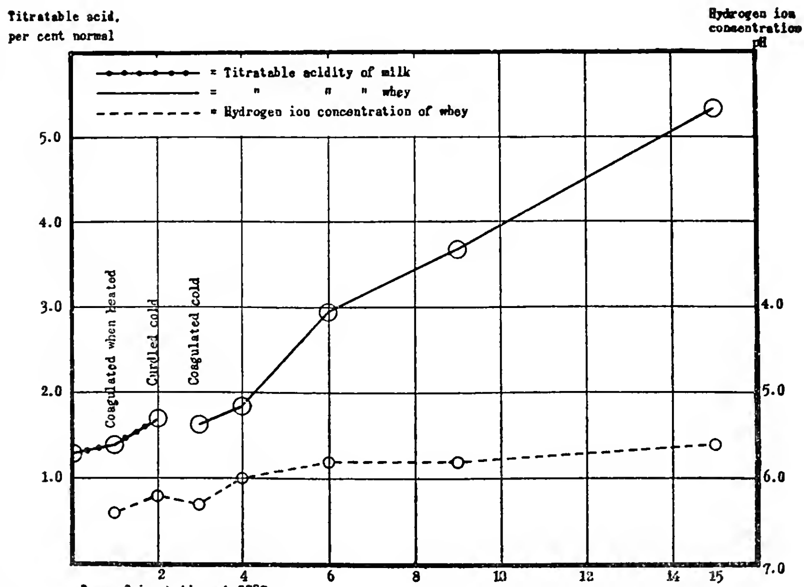
Strain No.	Sugar.	Relation to air.	Incubated 7 days.	
			Titration.	pH
			per cent	
X	Dextrose.	Not sealed.	3.9	5.1
	"	Sealed.	2.1	6.2
Sterile methylene blue.	"	"	Remained colorless.	

The titratable acidity is expressed as per cent normal acid.

It is to be noted that the behavior of *Bacillus pyogenes* as regards fermentation of sugars in relation to oxygen tension appears at variance with that of other organisms, notably those of the colon group. The idea, possibly correct in the case of most organisms, is prevalent that facultative anaerobic organisms are able to satisfy their oxygen requirements by breaking down fermentable sugars and that in such cases fermentation is likely to be more vigorous or at least more apparent under anaerobic conditions than in the presence of free oxygen. Some strains of *Bacillus coli* may produce an alkaline reaction in the bulb and acid and gas in the closed arm of the fermentation tube containing saccharose bouillon. *Bacillus cloacæ* may react similarly in lactose bouillon. The alkalinity or lower acidity of the bulb, however, may not be due to diminished fermentative activity but to the simultaneous production of large amounts of alkali.

Growth in Milk.

One of the cardinal cultural characteristics of *Bacillus pyogenes* is its ability to coagulate milk and slowly digest the curd. If an indicator solution such as rosalic acid and china blue (Bronfenbrenner, 1918–19) or bromocresol purple (Clark and Lubs, 1917, *b*) is placed in the milk, the latter is seen to become acid and remain acid for at least 3 weeks during which time much of the coagulum disappears. The question arises as to whether the coagulation of milk is due to acid or to an enzyme.



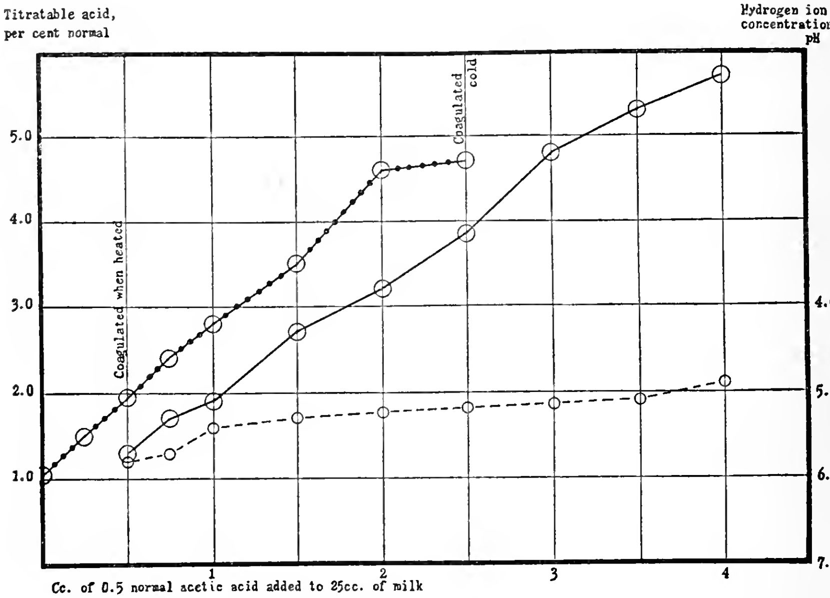
TEXT-FIG. 2. Titratable acidity and hydrogen ion concentration of fat-free milk after inoculation with *B. pyogenes*.

Seven tubes of fat-free milk were inoculated with *Bacillus pyogenes*. A tube was withdrawn for titration of total acidity and determination of hydrogen ion concentration[4] after incubation for 1, 2, 3, 4, 6, 9, and 15 days. The results are plotted in Text-fig. 2. The titratable acidity increased steadily. The hydrogen ion concentration of the whey

[4] Determinations of hydrogen ion concentration were made by the colorimetric method of Clark and Lubs (1917, *a*).

increased very little after the 6th day and reached a maximum of pH 5.6 in 15 days. The milk showed visible coagulation without application of heat on the 2nd day of incubation when the hydrogen ion concentration of the whey was pH 6.2 and the titratable acidity of the curdled milk was 1.7 per cent normal.

For comparison with these results tubes of milk were acidified with increasing amounts of 0.5 N acetic and hydrochloric acids. The results of titration of total acidity and determination of hydrogen ion concentration are plotted in Text-figs. 3 and 4.

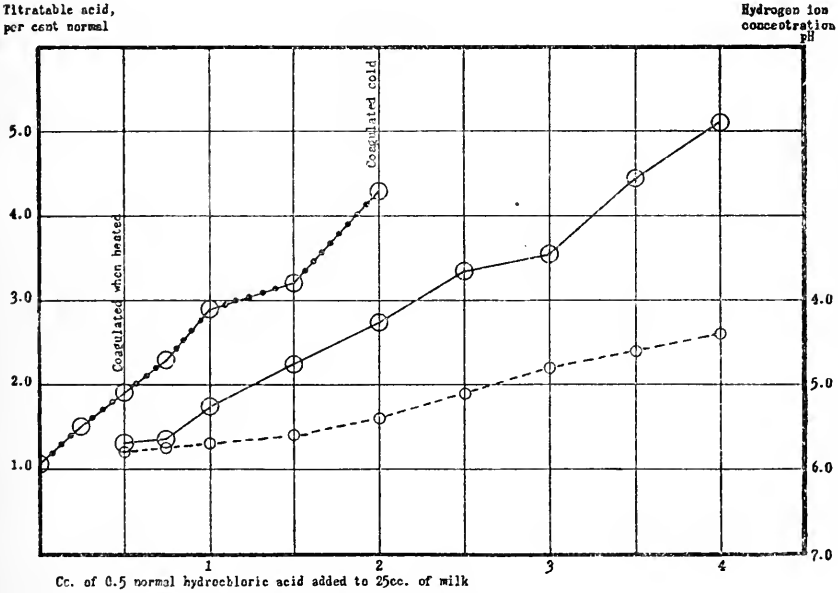


TEXT-FIG. 3. Total acidity and hydrogen ion concentration of fat-free milk after the tubes had been acidified with increasing amounts of acetic acid.

As was to be expected the hydrogen ion concentration showed more rapid increase with addition of hydrochloric acid than with similar amounts of acetic acid. With both the strong and the weak acid, however, the milk was coagulated without application of heat when it had reached a titratable acidity of 4.3 and 4.7 per cent normal respectively and the whey had a hydrogen ion concentration of pH 5.4 to 5.2. As was also to be expected the titratable acidity of the weak acid accompanying a hydrogen ion concentration sufficient to coagu-

late the milk was higher than that of the strong acid, the latter being more highly ionized. The difference is even more apparent in the titratable acidity of the whey.

In fermented bouillon plus 0.2 per cent of calcium chloride and 2.0 per cent of sodium caseinate *Bacillus pyogenes* produced a casein coagulum or sediment within 24 hours at 37°C. In the absence of calcium chloride no coagulation occurred. The same media gave similar results with rennet. These tests were made in media of four different reactions; namely, pH 6.2, 6.8, 7.1, and 7.7. After inoculation



TEXT-FIG. 4. Total acidity and hydrogen ion concentration of fat-free milk after the tubes had been acidified with increasing amounts of hydrochloric acid.

with *Bacillus pyogenes* and incubation for 20 hours, during which time coagulation occurred in all of those containing calcium chloride, the reactions were pH 6.2, 6.5, 6.7, and 7.2 respectively. The sedimented coagulum in the last tube was the most voluminous and the supernatant fluid from this tube gave no further precipitate when acidified with acetic acid. In the tube with pH 6.2 the sediment was more compact than in the others but acidification of the supernatant fluid showed that some casein was still in solution. It has also been found

that when *Bacillus pyogenes* is grown in fermented bouillon, plain bouillon, serum bouillon, dextrose bouillon, or dextrose serum bouillon plus sodium caseinate, the medium becomes very cloudy, almost opaque, in 24 hours and then clear again in another day or two. No sediment is formed or at most only a sediment of the organism itself. When the medium clears, however, the casein has disappeared and cannot be precipitated by acetic or nitric acid. Serum does not completely disappear and can be precipitated by nitric acid in cultures more than a week old. Apparently in the dextrose bouillon the casein is digested before the acidity rises sufficiently to precipitate it.

The preparation of casein calcium bouillon is attended with some difficulty. The method employed was as follows: To the sterile fermented bouillon add sufficient sterile 10 per cent calcium chloride to make a 0.2 per cent solution. Add sufficient sterile hydrochloric acid to dissolve the calcium phosphate which has been precipitated. At this point the medium reacts pH 6.2 to 6.4. Add sufficient 10 or 20 per cent sterile casein solution to make 2.0 per cent of casein in the bouillon. After the casein has been added the medium may be made alkaline if desired by the addition of sterile sodium hydrate. The calcium is apparently held by the casein so that it is not again precipitated as phosphate in the presence of a moderate amount of alkali. The preparation of this medium could probably be simplified by the use of calcium caseinate rather than sodium caseinate.

Since milk is coagulated by *Bacillus pyogenes* at a titratable acidity far below that required of hydrochloric or acetic acid and also at a much lower hydrogen ion concentration, and since soluble casein is coagulated in a neutral or slightly alkaline sugar-free medium, it appears that the coagulation of milk by *Bacillus pyogenes* is an enzyme rather than an acid coagulation. It is also to be noted that the casein is digested even in the presence of an excess of fermentable sugar. The process of digestion, however, seems to stop short of ammonia production since in a milk culture incubated for 3 weeks no increase in ammonia could be detected by Folin's method.

Immunological Study.

Four representative strains of *Bacillus pyogenes* were selected for the immunization of rabbits. Strain IV was selected because it showed slight morphological differences from the others. Strains VII, VIII, and X were selected because they were isolated from different lesions; *i.e.*, lungs, uterus, and a fetus, respectively. Rabbits

TABLE II.

Cross-Precipitation of Strains IV, VII, VIII, and X.

Strain No.	Serum No.	Dilutions.					Controls.	
		1:10	1:20	1:40	1:80	1:160	Normal rabbit serum, 1:10.	Salt solution.
IV	IV	++++	+++	++	++	+	–	–
	VII	++++	++	+	–	–	–	–
	VIII	++	Sl.	–	–	–	–	–
	X	++++	++	+	–	–	–	–
VII	IV	++	++	+	Sl.	–	–	–
	VII	++++	++++	++	–	–	–	–
	VIII	+	–	–	–	–	–	–
	X	++++	++	+	Sl.	–	–	–
VIII	IV	++	+	Sl.	–	–	–	–
	VII	++++	+++	+	Sl.	–	–	–
	VIII	++	+	+	+	Sl.	–	–
	X	+++	++	++	+	+	–	–
X	IV	++	+	Sl.	–	–	–	–
	VII	+++	++	+	+	–	–	–
	VIII	+	+	Sl.	–	–	–	–
	X	++++	+++	++	+	–	–	–

In the tables ++++ indicates maximum precipitation; Sl., slight precipitation.

were given at first several series of subcutaneous and intravenous injections of killed cultures, and later increasing amounts of living bouillon cultures intravenously at intervals of about 1 week. The rabbits tolerated the injections well and though all eventually succumbed to *Bacillus pyogenes* infection noticeable lesions did not develop until after the immune serum had been secured.

TABLE III.

Precipitation of All Strains by Sera IV and X.

Strain No.	Serum No.	Dilutions.					Controls.	
		1:10	1:20	1:40	1:80	1:160	Normal rabbit serum, 1:10.	Salt solution.
I	IV	++	+	Sl.	–	+	–	–
	X	+++	++	++	+	Sl.	–	–
II	IV	+++	+++	+	Sl.	–	–	–
	X	++++	+++	++	+	+	–	–
III	IV	++	+	+	Sl.	–	–	–
	X	++++	++	+	–	–	–	–
IV	IV	++++	+++	++	++	+	–	–
	X	++++	++	+	–	–	–	–
V	IV	++	++	+	–	–	–	–
	X	++++	+++	++	±	–	–	–
VI	IV	+++	++	+	–	–	–	–
	X	++++	++++	+++	++	+	–	–
VII	IV	++	++	+	Sl.	–	–	–
	X	++++	++	+	"	–	–	–
VIII	IV	++	+	Sl.	–	–	–	–
	X	+++	++	++	+	+	–	–
IX	IV	++	+	Sl.	–	–	–	–
	X	+++	++	+	–	–	–	–
X	IV	++	+	Sl.	–	–	–	–
	X	++++	+++	++	+	–	–	–
XI	IV	+	Sl.	–	–	–	–	–
	X	++++	++	+	–	–	–	–
XII	IV	++	++	+	Sl.	–	–	–
	X	++++	++++	+++	++	+	–	–

The titration of agglutinins was unsatisfactory because all but one or two strains were agglutinated to a considerable extent by normal rabbit serum in dilutions of 1:100 or 1:1,000. There was relatively little agglutination in salt solution controls. By regarding as positive only those tubes in which agglutination was stronger than in the normal serum controls it was evident that the immune sera produced agglutination of the homologous strains and many others in dilutions of 1:800 to 1:3,200. However, the normal agglutinins were such a disturbing factor that it is considered unsafe to draw any conclusions as to the relationship of the various strains on the basis of agglutination.

More satisfactory results have been obtained by titrating the precipitins. The precipitinogen used in the titrations consisted of the clear supernatant fluid obtained by centrifuging bouillon and blood bouillon cultures after incubation for 1 month. During incubation the cultures were frequently shaken. The blood bouillon and the plain bouillon yielded equally good precipitinogen. The precipitin titer of the sera was much lower than the agglutination titer but there was no precipitation in the controls and the results were quite definite. In Table II are given the results of cross-precipitation of the four strains employed for immunization of rabbits. There is precipitation of all the strains by each of the sera. Except in the case of Strain VIII the precipitinogen of each strain is precipitated best by the homologous serum. Serum VIII was rather a weak serum probably due to the fact that the rabbit from which it was obtained could not be injected regularly because of its having "snuffles." No great diversity among the strains is revealed by the precipitin titration. Strain IV, however, does seem to stand a little apart from the other three. Serum IV and Serum X were therefore selected for titration with all the strains. The results are recorded in Table III. The precipitinogens of all the strains except No. IV show greater precipitation with Serum X than with Serum IV. It appears that Strain IV is slightly different from the others immunologically as well as morphologically.

Pathogenicity for Rabbits.

According to Berger (1908), Holth (1908), and others, rabbits are the most susceptible of the small laboratory animals to experimental infection with *Bacillus pyogenes*. Guinea pigs are less susceptible, and mice least so.

In addition to the four rabbits used for immunization five others were injected intravenously with a single dose of 3 or 4 cc. of living bouillon culture, two with Strain IV and three with Strain X. Those immunized against Strains IV, VII, VIII, and X were repeatedly injected intravenously with these strains. Eight of the rabbits succumbed to the infection or were killed when in a badly crippled or moribund condition. The ninth may have died as a result of bleeding from the heart. There were no noticeable immediate symptoms following injections, no toxic symptoms, but usually a rise in temperature within 48 hours. Following this the rabbits appeared normal for 2 or 3 weeks. The first symptom of infection was often a progressive loss in weight followed by lameness or in four cases by paralysis. One of the paralyzed rabbits died during our absence from the laboratory and a complete autopsy was not obtained. The lesions found in the remaining seven rabbits may be classified as follows:

Lesions of bones in six rabbits (vertebræ three, femur four, rib one, tibia one).
Lesion of joint in one rabbit.
Lesions of muscles or tendons in three rabbits.
Lesion of lymph node in one rabbit.
Endocarditis in one rabbit.
Pneumonia in one rabbit.
Kidney abscesses in one rabbit.

All the above lesions were studied histologically and culturally, and were found to be due to the organism injected. A summary of these results is given in Table IV.

Rabbit J was injected subcutaneously only with bouillon cultures of Strain X at three different times as follows:

1st day. Injection A, 0.2 cc.; Injection B, 1.0 cc.
7th day. Injection C, 0.2 cc.; Injection D, 0.4 cc.; Injection E, 0.6 cc.
10th day. Injection F, 0.2 cc.; Injection G, 0.4 cc.; Injection H, 0.6 cc.
12th day. Rabbit chloroformed. Abscesses removed and fixed in Zenker's fluid. The rabbit had lost about 100 gm. in weight but was otherwise apparently well. At autopsy the subcutaneous lesions were the only ones found.

The abscesses at the time of removal were therefore 2, 5, and 11 days old. The youngest abscesses appeared grossly as flat discs about the size of five cent pieces and on gross section appeared to be composed of a fibrous tissue infiltrated with a small amount of yellowish

translucent viscid pus, and surrounded by soft hemorrhagic edematous tissue. The largest and one of the oldest abscesses, B, was firm and nodular, about 1 cm. in diameter, composed of a thick capsule of dense fibrous tissue enclosing a thick creamy yellow somewhat viscid pus.

A stained section of one of the youngest abscesses, H (Fig. 10), is roughly oval, about 7 mm. long by 2 mm. broad. It lies in a loose areolar connective tissue with a layer of transversely cut muscle fibers within 1 mm. of one side of the abscess. There is no definite capsule. The abscess is bordered by a thin layer of fibrin and necrotic connective tissue. The connective tissue on all sides is infiltrated by polymorphonuclear leucocytes while the perimysium of the adjacent muscular tissue contains many eosinophilic cells. There are also some large mononuclear cells many of which are doubtless fibroblasts though some may be endothelial leucocytes. Some of the muscle fibers nearest the abscess are invaded by polymorphonuclear leucocytes and are undergoing heterolysis. The contents of the abscess are principally a mass of degenerating polymorphonuclear leucocytes. Scattered about are bits of collagenic fibers, each embedded in a mass of *B. pyogenes* as though the bacilli are growing on the collagenic fibers as a medium (Fig. 14). Very few bacilli are found elsewhere than clustered about these fibers. About these individual masses of bacilli there is always an area of compact necrosed cells with few visible nuclei and in one part these areas by confluence have formed the beginning of the characteristic central zone of the older abscesses.

The older abscesses, A and B, are spherical and surrounded by dense fibrous capsules, 2 to 3 mm. thick (Fig. 11). The abscess is differentiated into three fairly distinct zones. The central zone (Fig. 11, *a*) is a ragged granular mass of dead and disintegrating cells with few visible nuclei. Within this zone many bacilli are scattered about, but towards its periphery these are in masses only a few of which still contain a fragment of collagenic fiber. Apparently these fibers are digested by the proteolytic action of the bacilli. Outside the central zone no bacilli are found. In the second or intermediate zone (Fig. 11, *b*) most of the cells have deeply stained nuclei which, however, exhibit pycnosis, caryorrhexis, or caryolysis. The outer zone (Fig. 11, *c*) resembles the central zone in general appearance. There are few visible nuclei, but no bacilli. It is bordered, however, by large mononuclear macrophages laden with nuclear and other cell debris.

In sections the bacilli are best stained by Gram's method used according to Holth's (1908) directions. Stirling's aniline gentian violet containing 1 or 2 per cent by weight of the dry stain has given us excellent results. To show the association of bacilli with collagenic fibers we have obtained excellent preparations with the following stains.

Orth's lithium-carmine, 20 minutes.

Acid alcohol, 3 minutes.

Stirling's aniline gentian violet, 10 seconds.

TABLE IV.

Rabbits Inoculated with B. pyogenes.

Rabbit.	Sex.	Strain No. Injection.	Maximum temperature.	Weight variation.	Localizations.	Result.
			°C.	gm.		
A	M.	IV. Repeated subcutaneous and intravenous injections for immunization.	40	1,890–2,355	Yellow spots in liver; hemorrhagic foci in lungs. (Incomplete autopsy.)	Died suddenly during night after being bled, 39th day after first injection of living culture.
B	"	VII. Repeated subcutaneous and intravenous injections for immunization.	41	1,650–1,865–1,380	Left femur; both kidneys; heart valve; epicardium.	Died after period of weakness 97th day after first injection of living culture.
C	"	VIII. Repeated subcutaneous and intravenous injections for immunization.	40.8	1,850–1,944–1,705	Muscle abscess in left quadriceps extensor femoris muscle. (Recurrent attacks of snuffles.)	Chloroformed 4½ mos. after first injection of living culture.
D	"	X. Repeated subcutaneous and intravenous injections for immunization.	40.2	1,650–2,010	3rd and 4th lumbar vertebræ; abscess surrounding and invading rib.	Paralysis of both hind legs. Chloroformed 5 mos. after first injection of living culture.
E	"	IV. 3 cc. intravenously.	39.8	1,960–2,125–1,945	(Incomplete autopsy.)	Paralysis of both hind legs and bladder. Died suddenly on 34th day after injection.

F	F.	X. 3 cc. intravenously.	41.2	1,935–1,980–1,040	Left femur.	Died 27 days after injection.
G	M.	IV. 4 " "	40.5	1,645–1,935–1,470	1st thoracic vertebra; right knee; both shoulder joints (periarticular abscesses).	Paralysis of left hind leg. Chloroformed on 81st day after injection.
H	"	X. 4 " "	41.4	1,835–1,895–1,240	Right femur; tendon of left gastrocnemius muscle; left instep; lungs.	Died on 13th day after injection.
I	"	X. 3 " "	39.8	1,815–1,910–1,240	1st, 2nd, and 3rd lumbar vertebræ; crest of right tibia; trochanter and gluteus minimus muscle of right femur; right zygomatic fossa; right biceps brachii muscle; left cubital gland.	Paralysis of both hind legs. Chloroformed on 64th day after injection.
J	"	X. Multiple subcutaneous injections.	39.4	3,580–3,415	Subcutaneous abscesses at points of injection.	Chloroformed 11th day after first injection.

Wash in water.
Lugol's iodine solution, 30 seconds.
Wash in water. Blot.
Decolorize in absolute alcohol.
Wash in water.
Mallory's aniline blue and orange G, 20 minutes.
Wash in water.
Decolorize and dehydrate in 95 per cent and absolute alcohol.
Xylol. Mount in balsam.

By the above method *B. pyogenes* is stained purple, collagenic fibers bright blue, leucocytes and tissue cell nuclei red or deep orange, blood, fibrin, and muscle yellow.

Of the lesions resulting from the intravenous injection of cultures a few deserve brief description. The most frequently produced lesions were those of the bones. Such lesions have been reported by Berger (1908), Holth (1908), and Koske (1906). Their protocols show that they encountered paralyses also in experimentally infected animals. Koske, unable to find lesions in the cords of paralyzed pigs, considered it possible that a specific neurotoxin might be involved but was unable to obtain toxic effects with filtered cultures. He does not mention examining the vertebræ of these animals. Four times we encountered paralyses in injected rabbits. In the three that were thoroughly autopsied were found lesions on the ventral floor of the spinal canal. These lesions were not visible from the ventral side of the spinal column and in fact could not be found until the cord had been removed. There were then found abscesses exerting pressure against the ventral side of the cord. In no case was the dura penetrated nor were the meninges infected. In the case of Rabbit G an abscess lay between the dura and the vertebral periosteum without invading the body of the vertebra. In Rabbits D and I were found intervertebral abscesses obliterating the intervertebral cartilages, invading the bodies of the vertebræ, and eroding the bone with more or less destruction of the floor of the spinal canal (Fig. 12).

In a stained section there is found proliferation of connective tissue about the abscess. The center of the focus is composed of a mass of disintegrated cells and nuclei which appear to have been small mononuclear cells rather than polymorphonuclear leucocytes, differing in this respect from the abscesses in the subcutis and other soft parts. In the proliferating fibrous tissue bordering and sometimes sur-

rounding the abscesses in the vertebræ are many large mononuclear cells (Fig. 15). Stained by Gram's method many of these large cells are seen to be filled with *B. pyogenes* (Fig. 16). Holth regards these cells as young fibroblasts rather than endothelial cells. In the necrotic center of the abscess bacilli lie scattered about.

The lesion here described is much like the "*grösseren Knoten*" described by Holth and found by him in various parts of the body—lungs, subcutis, peritoneum, etc. He describes masses of "*Rundzellen*" in the center of the abscess undergoing necrosis and disintegration, an intermediate zone of tissue resembling the round cell masses of smaller abscesses, and an outer capsule of connective tissue interrupted by small masses of round cells. As the tumor grows the round cell masses fuse and the process of disintegration advances. He then describes the bacilli scattered about in the center of the abscess and within what he regards as large connective tissue cells of the capsule. He does not mention the presence of polymorphonuclear leucocytes in the abscess, whereas we found them to predominate in abscesses of soft parts. It is to be noted, however, that because of pycnosis these cells often resemble round cells. It is to be noted also that in the subcutaneous abscesses described above no bacilli were found in the cells of the capsule, possibly because the abscesses studied by us were not old enough.

Lesions in the long bones—femur and tibia (Fig. 13)—were fundamentally like those in the bodies of the vertebræ, modified by the tissues encountered. The predominating cells of the reaction resembled plasma cells. In places there was marked proliferation of connective tissue. In places the bacilli appeared to grow freely among the cells of the bone marrow. Occasionally bone lacunæ were seen filled with masses of bacilli. There was always erosion of the bone making it quite porous. In two cases the femur broke under very little strain as the rabbits were being tied out for autopsy. A nodular encapsulated abscess enveloped the rib of Rabbit D eroding it from without. The abscess may have been subperiosteal in origin.

The knee of Rabbit G contained a glairy viscid pus and one of the joint surfaces was eroded.

In Rabbits C, H, and I true myositis and tendinitis were encountered. In these cases the muscle fibers had been completely heterolyzed or digested and the muscle converted into a closed sac of viscid

glairy pus within the epimysium (Fig. 9). There were present in the pus and perimysium plasma cells, endothelial leucocytes, neutrophilic polymorphonuclear leucocytes, and, especially conspicuous, eosinophilic leucocytes. Bacilli were abundant in all but the oldest lesions.

The diseased mitral valve of Rabbit B was encrusted with masses of *Bacillus pyogenes*, leucocytes, and necrotic tissue. The tissue of the valve was largely fibrin.

In the pneumonic lung of Rabbit H masses of bacilli were found beneath the pulmonary pleura. It appeared that growth had started from bacilli lodged in the pleural capillaries. The alveoli were filled with blood, fibrin, and desquamated epithelium.

The kidney abscesses of Rabbit B were in the form of pyramids with bases at the cortex. Around the borders of the abscesses the glomeruli and the capillaries of the interstitial tissue were plugged with leucocytes and masses of bacilli. The interlobular arteries were similarly plugged.

Resemblance to Other Organisms. Classification.

The close resemblance of *Bacillus pyogenes* to streptococci has been mentioned above. This is especially true of some strains when grown on certain media, a resemblance so close that the bacteriologist working with milk or animal diseases must be on his guard not to confuse them. *Bacillus pyogenes* produces laking of a suspension of blood or blood corpuscles in salt solution as do the hemolytic streptococci of human origin (Brown, 1920). Its limiting hydrogen ion concentration in dextrose bouillon would also mislead one to place it among the human streptococci (Avery and Cullen, 1919). On the other hand, *Bacillus pyogenes* liquefies coagulated blood serum and is distinctly diphtheroid at times. Morphologically, therefore, it may be one of the diphthero-streptococci now and then described. Such organisms, are occasionally isolated from human lesions, especially pneumonias and though *Bacillus pyogenes* has never been identified in man, it would be well for bacteriologists to keep it in mind. Since it is found in several species of animals and is widely disseminated in milk it may be that there are rare cases of human infection.

There is also found in animals a group of small Gram-positive organisms closely resembling *Bacillus pyogenes* morphologically and culturally. We have studied two such strains from the livers of calves and one from the lung of a hog. The strains from calves were apparently alike, and had the same fermentation reactions as *Bacillus pyogenes*. They were cultivated with greater difficulty than the latter, however. They grew best under partially anaerobic conditions and would not grow in serum-free media. There was no apparent growth in milk. Gelatin and coagulated serum were not liquefied and growth on the latter was scarcely visible. There was very little laking of horse blood in agar plates. A rabbit was repeatedly injected intravenously with several cubic centimeters of serum bouillon cultures of one of these strains with no ill effects. Serum from this rabbit precipitated precipitinogens of both of these strains in dilution of 1:80 or 1:160 but produced no precipitation of the precipitinogens of strains of *Bacillus pyogenes*. Neither did *Bacillus pyogenes* antisera precipitate precipitinogens of these two strains.

A strain from a hog also resembled *Bacillus pyogenes* morphologically. It differed from *Bacillus pyogenes* and the other two strains just described in that it fermented salicin. It resembled the two strains from calves in failing to liquefy gelatin or coagulated serum but grew much better than those strains. No visible change was produced in milk but sufficient acid was produced so that when the culture tube was placed in boiling water the milk was coagulated. An indefinite zone of hemolysis was produced in the blood agar plate.

Glage (1913) points out a certain resemblance of *Bacillus pyogenes* to the bacillus of swine erysipelas. Morphology, growth in gelatin, and the lesions produced in hogs and small experimental animals serve to differentiate the two.

Dunkel (1908) thought that *Bacillus pyogenes* could be transformed into *Bacillus pseudotuberculosis ovis* by animal passage and therefore regarded the two as of the same species. The two are certainly not alike when studied as isolated from their respective hosts. The dry colonies described as characteristic of *Bacillus pseudotuberculosis*, pigmented colonies on coagulated blood serum, and failure to produce change in milk are not at all like *Bacillus pyogenes*. There may be some morphological resemblance.

Priewe (1911) and Glage (1913) have claimed that *Bacillus pyogenes* belongs to the influenza bacillus group for the following reasons: hemoglobinophilic habit, form, size, non-motility, lack of spores, abundance in green pus, growth at high temperature only, and slight virulence for small laboratory animals. Priewe claimed that a *Bacillus pyogenes* antiserum agglutinated *Bacillus influenzæ.* We have not tried to repeat the latter observation. However, we do regard it as well established that *Bacillus pyogenes* is Gram-positive and that it is not hemoglobinophilic. The other characters enumerated by the above authors are common to so many and dissimilar organisms that they are of little value as evidence of relationship of these two organisms. *Bacillus pyogenes* does not have the foul odor characteristic of *Bacillus influenzæ* and the two do not produce similar lesions in rabbits. Grips (1903), Grips, Glage, and Nieberle (1904), and Priewe (1911) have regarded swine-plague as due primarily to *Bacillus pyogenes* with *Bacillus suisepticus* as a secondary invader. They regard the primary infection as a "*Tierinfluenza.*" The views of these authors have not been accepted by others. Olt (1904) has criticized them thoroughly but asserts that many of the lesions characteristic of chronic swine-plague are due to *Bacillus pyogenes.*

The forms assumed by *Bacillus pyogenes* bear a striking resemblance to those of *Asterococcus mycoides* described by Borrel, Dujardin-Beaumetz, Jeantet, and Jouan (1910) as the cause of bovine pleuropneumonia. The latter organism is, however, much the smaller. The work of the authors mentioned is almost wholly morphological and the result of staining by Gram's method is not mentioned. Buchanan (1918), however, places *Asterococcus* in the Gram-negative subtribe Hemophilinæ. The other genus of this subtribe is *Hemophilus,* of which the type species is *Hemophilus influenzæ,* the influenza bacillus of Pfeiffer. Since Buchanan and Murray (1916) describe *Bacillus pyogenes* as a member of the hemophilic or influenza group they presumably classify it also in the subtribe Hemophilinæ where it can hardly belong in view of its being Gram-positive. Preisz (1906) regarded *Bacillus pyogenes* as one of the "*Corynebakterien.*" We are more inclined to place it in the genus *Corynebacterium* as defined in the Preliminary Report of the Committee of the Society of American Bacteriologists on Characterization and Classification of Bacterial Types (1917) than in the so called influenza group.

SUMMARY.

Bacillus pyogenes is probably quite common in this country, as it is known to be in Europe.

A careful study of twelve strains from cattle and one from a hog has disclosed the following characteristics which have not been reported or have been in dispute.

Bacillus pyogenes is Gram-positive and pleomorphic, producing forms ranging from short chains of streptococcoid elements to branching filaments.

It is hemolytic, producing the beta type of hemolysis in blood agar. It is not hemoglobinophilic, though its growth is greatly favored by some higher protein material such as egg albumin, serum, or blood.

It ferments xylose in addition to the substances previously reported.

The coagulation of milk by *Bacillus pyogenes* is primarily an enzyme coagulation and the subsequent digestion of the curd takes place in an acid medium.

The intravenous injection of rabbits was invariably fatal. The lesions most commonly developed were those of the bones. Paralysis was frequently produced, and in each case was caused by lesions in the vertebræ exerting pressure against the ventral columns of the spinal cord. Muscle abscesses were also frequently produced.

The authors regard the organism as belonging to the *Corynebacteria* rather than to the influenza group.

BIBLIOGRAPHY.

Avery, O. T., and Cullen, G. E., The use of the final hydrogen ion concentration in differentiation of *Streptococcus hæmolyticus* of human and bovine types, *J. Exp. Med.*, 1919, xxix, 215.

Berger, E., Bakteriologische Untersuchungen über einige chronische Lungenentzündungen des Rindes, *Z. Infektionskrankh. Haustiere*, 1908, iii, 356.

Borrel, Dujardin-Beaumetz, Jeantet, and Jouan, Le microbe de la Péripneumonie, *Ann. Inst. Pasteur*, 1910, xxiv, 168.

Bronfenbrenner, J., A new indicator for direct reading of hydrogen ion concentration in growing bacterial cultures, *J. Med. Research*, 1918–19, xxxix, 25.

Brown, J. H., The use of blood agar for the study of streptococci, Monograph of The Rockefeller Institute for Medical Research, No. 9, New York, 1919.

Brown, J. H., The cultural differentiation of beta hemolytic streptococci of human and bovine origin, *J. Exp. Med.*, 1920, xxxi, 35.

Buchanan, R. E., and Murray, C., Text book of veterinary bacteriology, Philadelphia, 2nd edition, 1916.

Buchanan, R. E., Studies in the nomenclature and classification of the bacteria. V. Subgroups and genera of the *Bacteriaceæ*, *J. Bacteriol.*, 1918, iii, 27.

Clark, W. M., and Lubs, H. A., The colorimetric determination of hydrogen ion concentration and its applications in bacteriology, *J. Bacteriol.*, 1917, *a*, ii, 1, 109, 191.

Clark, W. M., and Lubs, H. A., A substitute for litmus for use in milk cultures, *J. Agric. Research*, 1917, *b*, x, 105.

Davis, D. J., Food accessory factors (vitamins) in bacterial culture with especial reference to hemophilic bacilli I, *J. Infect. Dis.*, 1917, xxi, 392.

Dunkel, H. P., Inaugural dissertation, Untersuchungen über die Beziehungen des *Bacillus pyogenes bovis* et *suis* zu dem *Bacillus pseudotuberkulose ovis*, Hildesheim, 1908.

Eggink, B., Inaugural dissertation, Aetiologie der Metritis beim Rinde, Sneek, 1914.

Glage, F., Ueber den *Bazillus pyogenes suis* Grips, den *Bazillus pyogenes bovis* Künnemann und den bakteriologischen Befund bei den chronischen, abszedierenden Euterentzündungen der Milchkühe, *Z. Fleisch- u. Milchhyg.*, 1902–03, xiii, 166.

Glage, F., Die Eiterungen bei den Haustieren, in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1913, vi, 172.

Grassberger, R., Beiträge zur Bakteriologie der Influenza, *Z. Hyg. u. Infektionskrankh.*, 1897, xxv, 453.

Grips, W., Ueber eine mit multipler Abzessbildung verlaufende Pleuritis und Peritonitis der Schweine und deren Erreger, *Z. Fleisch- u. Milchhyg.*, 1898, viii, 166.

Grips, W., Zur Aetiologie der Schweineseuche, *Deutsch. tierärztl. Woch.*, 1903, xi, 185.

Grips, W., Glage F., and Nieberle, C., Die Schweineseuche, *Fortschr. Vet.-Hyg.*, 1904, ii, 5.

Holth, H., Untersuchungen über den *Bacillus pyogenes* und die durch ihn hervorgerufenen Gewebsveränderungen, *Z. Infektionskrankh. Haustiere*, 1908, iii, 155.

Koske, F., Die Beziehungen des *Bacillus pyogenes suis* zur Schweineseuche, *Arb. k. Gsndhtsamte*, 1906, xxiv, 181.

Künnemann, O., Ein Beitrag zur Kenntniss der Eitererreger des Rindes, *Arch. wiss. u. prakt. Thierheilk.*, 1903, xxix, 128.

Lucet, A., Recherches bactériologiques sur la suppuration chez les animaux de l'espèce bovine, *Ann. Inst. Pasteur*, 1893, vii, 325.

Olt, Ueber die pyæmische Kachexie der Schweine und die Schweineseuche, *Deutsch. tierärztl. Woch.*, 1904, xii, 325.

Olt, Ueber das Vorkommen des *Bacillus pyogenes* als Sputumbakterium und Eitererreger bei verschiedenen Tierarten, *Deutsch. tierärztl. Woch.*, 1908, xvi, 617.

Poels, Rapport over de Kalverziekte in Nederland, The Hague, 1899.

Preisz, H., Vizsgálatok a sertésvész aetiologiája körül, *Allatorvosi lapok*, 1906, xxix, 325, 339, 350, 360.

Priewe, W., Inaugural dissertation, Die Stellung des Grips'-schen Bazillus im System der Bakterien, Borna-Leipzig, 1911.

Pütz, H., Der *Bacillus pyogenes* und seine Beziehungen zur Schweinesuche, *Z. Fleisch- u. Milchyg.*, 1904, xiv, 361.

Smith, T., and Brown, J. H., A study of streptococci isolated from certain presumably milk-borne epidemics of tonsillitis occurring in Massachusetts in 1913 and 1914, *J. Med. Research*, 1914–15, xxxi, 455.

Tuff, P., Foredrag holdt. i den danske Dyrlägeforening d. 24. Febr. 1906, referred to in *Maanedsskr. Dyrläger.*, 1906, xviii.

Ward, A. R., Suppuration in cattle and swine caused by *Bacterium pyogenes*, *Cornell Vet.*, 1917, *a*, xii, 29.

Ward, A. R., *Bacterium pyogenes* and its relation to suppurative lesions in animals, *J. Bacteriol.*, 1917, *b*, ii, 619.

Winslow, C.-E. A., Broadhurst, J., Buchanan, R. E., Krumwiede, C., Jr., Rogers, L. A., and Smith, G. H., The families and genera of the bacteria. Preliminary Report of the Committee of the Society of American Bacteriologists on Characterization and Classification of Bacterial Types, *J. Bacteriol.*, 1917, ii. 505.

EXPLANATION OF PLATES.

Plate 14.

Fig. 1. A horse blood agar plate culture of *B. pyogenes* X after incubation for 48 hours. The large round hemolyzed area was produced by a few particles of saponin.

Plate 15.

Gram stains. Magnification × 1,000.

Fig. 2. The bacillary form of Strain X.

Fig. 3. The fusiform form of Strain IV.

Fig. 4. The fusiform form of Strain X.

Fig. 5. The filamentous or branching form of Strain IV.

Fig. 6. The diphtheroid form of Strain X showing a few buds.

Fig. 7. The streptococcoid form of Strain IV.

Fig. 8. The streptococcoid form of Strain X.

Plate 16.

Fig. 9. Rabbit H. Abscess in tendinous end of gastrocnemius muscle at *a*. Above the abscess is a section of the distal end of the flexor digitalis pedis sublimis muscle. Eosin and methylene blue stain. × 10.

Fig. 10. Rabbit J. Subcutaneous abscess H, 48 hours after injection. The capsule and zonal arrangement of the abscess not yet developed. Gram and aniline blue stain. × 5.

Fig. 11. Rabbit J. Subcutaneous abscess A, 11 days after injection. A very thick fibrous capsule (*d*) about the abscess and the three zones (*a*, *b*, *c*) of the abscess described in the text are shown. Eosin and methylene blue stain. × 5.

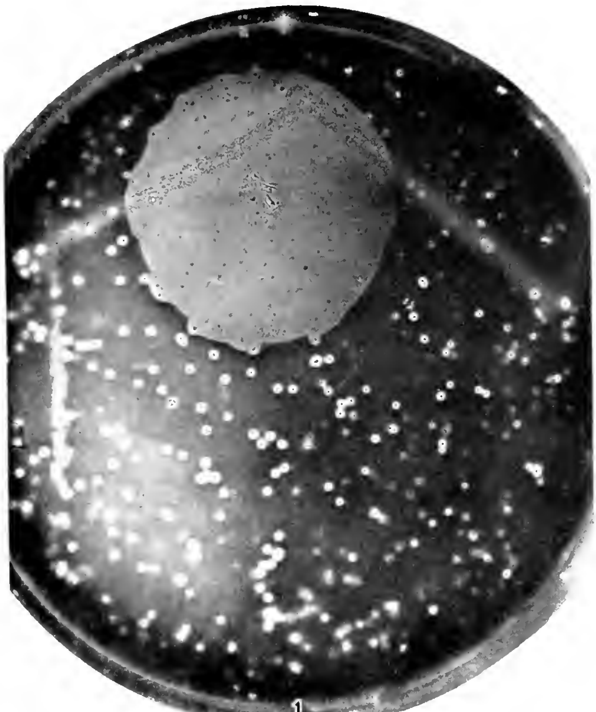
Fig. 12. Rabbit D. Abscess occupying the body of the third lumbar vertebra at *a*, the spinous process and dorsum of the spinal foramen having been removed. Only a small bony fragment of the floor of the spinal canal remains at *c*. Eosin and methylene blue stain. × 5.

Fig. 13. Rabbit I. Abscess in crest of tibia at *a*. Transverse section. Eosin and methylene blue stain. × 10.

Fig. 14. Rabbit J. A field from the center of the abscess shown in Fig. 10, showing the minute bacilli clustered about remnants of connective tissue fibers. Gram and aniline blue stain. × 430.

Fig. 15. Rabbit D. A field from the region marked *b* in Fig. 12, showing large mononuclear cells probably on the border of an abscess. Eosin and methylene blue stain. × 430.

Fig. 16. Rabbit D. A field from the same region as Fig. 15, showing the large mononuclear cells filled with *B. pyogenes*. Gram stain. × 430.



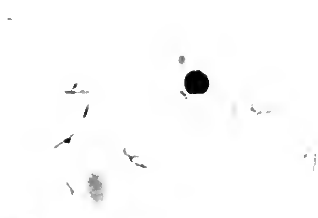
1

(Brown and Orcutt: *Bacillus pyogenes*.)

2

3

4



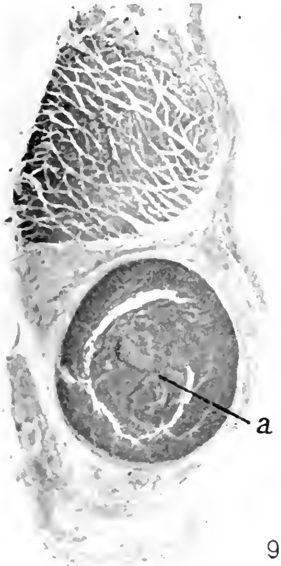
5

6

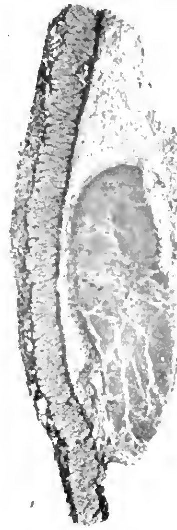
7

8

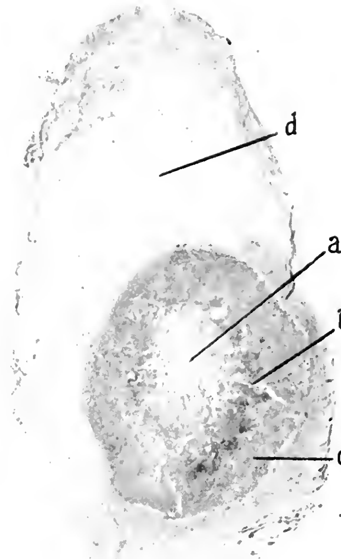
(Brown and Orcutt: *Bacillus pyogenes*.)



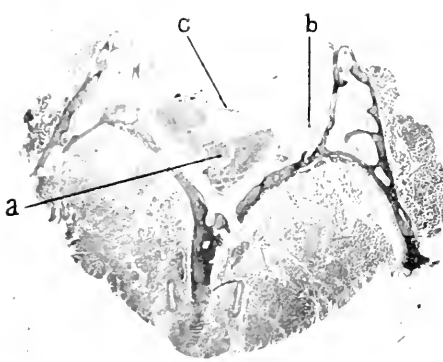
9



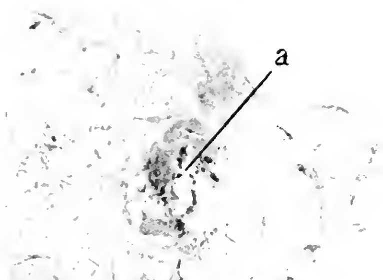
10



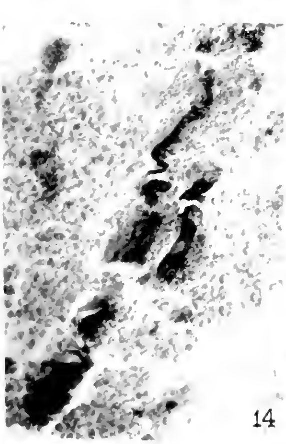
11



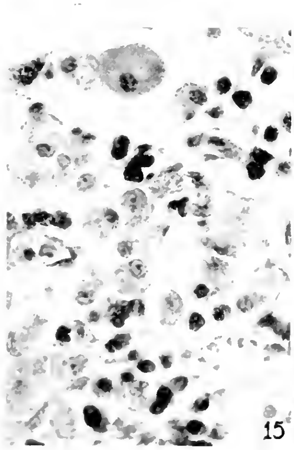
12



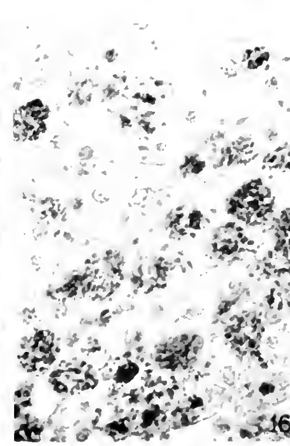
13



14



15



16

(Brown and Orcutt: *Bacillus pyogenes*.)

THE BILIARY FACTOR IN LIVER LESIONS.

By PEYTON ROUS, M.D., and LOUISE D. LARIMORE, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 17 to 22.

(Received for publication, April 5, 1920.)

The qualification "biliary" has long been applied to a diverse group of hepatic lesions distinguished by a peculiar cirrhosis with more or less evidence of stasis in the finer bile ducts and inflammation of their walls. The actual part played by bile in the production of the connective tissue changes here seen and of chronic lesions in general of the human liver is not definitely known. According to some authors human bile is incapable of causing any permanent hepatic injury. If this be true man differs from all other well studied animals, for in them without exception aseptic bile stasis results in important local changes. There is positive evidence, too, that human bile may on occasion be the cause of important damage to the liver. Sudden total obstruction of the common duct results in the so called icteric necroses,[1] which may attain to the size and character of "biliary infarcts,"[2] large wedge-shaped areas of bile-stained necrosis such as regularly follow obstruction in rabbits and guinea pigs. Long continued stasis, when uncomplicated by infection, as is rarely the case, has been known to result in stellate cirrhosis with many new-formed bile ducts, a lesion found in the rabbit under like circumstances;[3] and, as in this animal, complete local stasis leads to liver atrophy.[4] Further examples might be cited. For the purposes of the present work we shall assume that human bile, while innocuous as compared with that of certain other species, can produce liver injury. Our

[1] Eppinger, H., *Beitr. path. Anat. u. allg. Path.*, 1902, xxxi, 230.

[2] Carnot, P., and Hervier, P., *Arch. méd. exp. et anat. path.*, 1907, xix, 76.

[3] Rolleston, H. D., Diseases of the liver, London, 2nd edition, 1912.

[4] Brissaud, E., and Sabourin, C., *Arch. physiol. norm. et path.*, 1884, iii, series 3, 345.

aim has been to obtain through experiment a better understanding of "biliary" lesions with special reference to the share of bile in their causation. No attempt will be made to ignore the factor of infection, which without doubt has for many instances a prime importance.

Choice of Animals.

The differences in the commoner laboratory animals as regards amount of the bile and its local effects have been admirably set forth by Quincke and Hoppe-Seyler.[5] The years since their summary have added little that is new. The rabbit and guinea pig respectively secrete nearly seven and eight times as much bile as the dog, and ten and twelve times as much as the cat. Man's rate of secretion places him near to the last mentioned animals. In them the constitutional effects of total biliary obstruction are more marked than the local, while in man death usually occurs of toxemia before cirrhotic changes are manifest.[3] In the guinea pig and rabbit, on the contrary, closure of the common duct results in fulminant liver destruction with cirrhosis, and the death of the animal, which follows after a few weeks at most, is traceable to this cause. The development of lesions following local duct obstruction is far more rapid than in the dog and cat, changes that may take a twelve-month in these latter[6] requiring only a fortnight or less in the rabbit. And in the rabbit, furthermore, secondary infection occurs but rarely, whereas in the dog it is so frequent a confusing factor as to have balked many investigators. On the basis of recent experience we have no hesitation in asserting that had dogs instead of rabbits been employed for the present work years instead of months would have been required to obtain an understanding of the simpler principles ruling biliary lesions—principles that are of evident general application, and to be derived as surely from observations upon one mammalian species as another.

[5] Quincke, H., and Hoppe-Seyler, G., in Nothnagel, H., Specielle Pathologie und Therapie, Vienna, 1899, xviii, 59.

[6] Harley, V., and Barratt, W., *J. Path. and Bacteriol.*, 1901, vii, 203.

Method.

In work already reported,[7] we have taken advantage of the singular fitness of rabbits for experiments upon the liver which in this species consists of two well separated masses, each with its own bile duct and blood vessels. The smaller, or lobe mass, as it may be called, forming approximately one-quarter of the whole --23.7 per cent after ablation of the caudate lobe (4 per cent)—will accommodate on emergency the entire portal stream and maintain the animal in good health when the larger mass, or main liver (72.3 per cent), is ablated. The liver of the rabbit adapts itself to new conditions far more rapidly than that of the dog or cat. Thus, for example, the gradual parenchymal atrophy and hypertrophy which follow local portal diversion require but 2 months in the rabbit for their approximate completion, whereas in the dog several times this period is required.[7] There exists, however, a very considerable drawback to the clinical study of jaundice in the rabbit in the fact that the bile pigment of the animal reacts but poorly to the ordinary color tests. Our work has not been of a sort to make this difficulty felt. A second and serious potential objection to rabbits is to be found in the intercurrent hepatic cirrhosis prevalent in certain localities. The lesion has been rare in our experience as in that of some others,[8] and individuals showing it at operation, the first step in every experiment, have been discarded. As a further control to the condition of the liver, the caudate lobe has regularly been ablated for section. The total bulk of the organ is thus reduced by about 4 per cent, as already stated.

The rabbits weighed from 1,400 to 2,500 gm. The general method of operation has been described in another connection.[7] Bile ducts to be ligated were dissected free of the surrounding structures and tied twice with fine black silk, but not cut, since this precaution against a restoration of continuity was found unnecessary. The ducts are extremely delicate. Almost all the postoperative mortality was due to rupture of them above the ligature. When a branch of the portal vein was to be tied, it was dissected out. Special care was always taken to avoid injury to the hepatic artery, for this in the rabbit usually results in more or less widespread necrosis. Recovery from operation was in general uneventful. For the better analysis of the lesions most of the animals were killed early, with chloroform or by bleeding from the heart. Bits of the liver tissue were placed upon agar and in bouillon, and additional stroke and loop inoculations were made. Infection was rare, and instances showing it were ruled from consideration. A scattered, localized coccidiosis was fairly frequent, however. It had no evident complicating effect upon the liver changes.

Zenker's fluid, formaldehyde, and alcohol were used as fixatives.

---

[7] Rous, P., and Larimore, L. D., *J. Exp. Med.*, 1920, xxxi, 609. All operations were performed under ether anesthesia.

[8] Grover, A. L., *J. Am. Med. Assn.*, 1915, lxiv, 1487.

The amount of the bile and the pressure developing within obstructed ducts are both relatively great in well fed animals.[9] To this circumstance and to differences in the character of the food[10] are probably attributable most of the considerable quantitative variations that may be observed in lesions resulting from a single procedure. Our animals were not fed for 18 hours prior to operation, but immediately thereafter they were placed upon the mixed diet of normal rabbits.

Results of Total Obstruction.

Nearly all attempts to obtain by experiment an understanding of biliary lesions, from Leyden's time on, have centered about the production of total stasis by ligation of the common duct; and there exist many descriptions of the ensuing changes.[11] Our observations have but confirmed the general findings, while yielding an interpretation of them that has proven productive.

The rabbit secretes bile so copiously—136.8 gm. per kilo of animal in 24 hours, according to Heidenhain[10]—that obstruction of the common duct is followed practically at once by marked distension of the passages. Within a few hours bile-stained parenchymal necroses appear, of all sizes, from those that involve but a single cell to wedge-shaped areas, the so called biliary infarcts, 0.5 cm. or more in diameter. The larger ones are stained a bright yellow-green and frequently show hemorrhages. All are obviously the result of direct extravasation of the bile, either within the lobule or at its periphery where the smallest collecting ducts have ruptured. An identical rupture at this latter point, the weakest one in the collecting system, occurs on the injection under pressure of fluid into the common duct.

Within a few days dilatation of the larger ducts becomes great, and pericholangitis develops, followed soon by a spreading, stellate cirrhosis. The changes are most marked in the region of the larger portal spaces, but there is active proliferation of the connective tissue throughout Glisson's capsule, and every lobule is soon enclosed and encroached upon peripherally. The smaller necroses are replaced by proliferation of the neighboring liver cells, but with the larger

---

[9] Bürker, K., *Arch. ges. Physiol.*, 1900–01, lxxxiii, 241.

[10] Heidenhain, R., in Hermann, L., Handbuch der Physiologie, Leipsic, 1883, v, pt. 1, 256. Hooper, C. W., and Whipple, G. H., *Am. J. Physiol.*, 1916, xl, 332.

[11] Richardson has an excellent brief description (Richardson, M. L., *J. Exp. Med.*, 1911, xiv, 401). For one more extensive, with a survey of the literature, see Fiessinger, N., and Roudowska, L., *Arch. méd. exp. et anat. path.*, 1914–15, xxvi, 18.

this is impossible, owing to involvement of all the lobular elements, and organization takes place by a connective tissue invasion. The surviving parenchyma becomes more and more jaundiced, and numerous intralobular bile thrombi appear. Small scattered necroses continue to develop, but no large ones form after the first days. The lobules become very irregular and diminish in size through the encroachment of connective tissue, and the latter rapidly penetrates in thin strands between the cell cords to the central vein. Within 3 weeks the liver has become a roughly hobnailed organ, with ducts everywhere greatly distended, and a cirrhosis that is at once periportal, or, more properly speaking, pericholangitic, unilobular, irregularly intralobular where necroses have been replaced, and almost regularly intralobular here and there where the connective tissue penetration is diffuse. Throughout the older cirrhotic tissue are great numbers of new-formed bile ducts. Despite the extensive parenchymal destruction, the liver remains of about the normal size. Thrombosis of portal branches not infrequently occurs, with some resultant atrophy of liver parenchyma, while elsewhere an hypertrophy may be observed when the condition of the animal permits, as is rarely the case. Always there is great emaciation, and death usually ensues within 4 or 5 weeks. Jaundice appears after the first few days of obstruction.

No human lesion resembles this one more than remotely, and attempts to utilize it in explanation of the biliary cirrhoses of man have been almost given up. Yet when its histologic components are considered separately the fact becomes plain that certain of them find their replicas in one kind of human liver cirrhosis, and certain in another. In a word, the rabbit lesion is mixed, involving injury from stasis throughout the entire length of the biliary tract. By altering the conditions it is possible, as we shall show, to localize the injury of stasis to regions corresponding to ducts of a single level or order, and thus to obtain cirrhoses of uncomplicated type which closely resemble the biliary cirrhoses of man.

Results of Local Obstruction.

The results of tying off the bile duct to the main liver mass of the rabbit, some three-fourths of the entire organ, while leaving that to the lobe mass free have been briefly described by Nasse.[12] We have repeated his experiments and can confirm his statements as regards the early changes, these alone having importance for the present work.

[12] Nasse, *Verhandl. deutsch. Ges. Chir.*, 1894, xxiii, pt. 2, 525.

The effects of the sudden stasis on the main liver, though outspoken, are far less marked than after total obstruction. Dilatation of the bile ducts and gall bladder is relatively moderate, biliary necroses are small and appear only during the first few days, and the animal remains in good condition, never becoming jaundiced. The occluded parenchyma shows no bile tinting after the 1st week. Cirrhosis makes its appearance gradually and differs from that of total stasis in its slow, orderly progress, almost complete failure to invade the lobuli, and, in the subordination of stellate growth about the larger ducts, to a diffuse interlobular proliferation (Fig. 1). But new-formed bile ducts are still a prominent feature. The parenchyma undergoes a rapid atrophy. Within a few weeks the mass is much smaller, diffusely cirrhotic, and relatively bloodless; and by the end of 4 months it is reduced to a tag from which parenchyma and new-formed bile ducts have alike disappeared.[12] There is only the slightest hobnailing at any time. As atrophy of the main liver progresses the lobe mass undergoes hypertrophy and eventually reaches the size of the entire normal organ.

The cirrhosis here approaches the pure unilobular in type, for penetration between the cell cords is almost wholly absent (Fig. 1). But there still exists the complication of a stellate periportal proliferation, with notable dilatation and pericholangitis of the larger ducts. Nasse attributed the atrophy to closure of the secretory channels of the liver, likening the result to that of ureteral ligation. This though, is no sufficient explanation, since under the conditions of total obstruction the liver holds its size, and there may even be some local hypertrophy despite the progressive general emaciation. With local obstruction similar attempts at repair are never seen, although the animal stays in good condition.

The Factor of Portal Obstruction.

It has seemed to us probable that the mildness of the biliary lesions, including the cirrhosis, after local obstruction, and the parenchymal atrophy as well, are due in great part to a single factor, namely, partial deflection of the portal stream from the affected region. Several facts support the view. When the duct from the main liver is tied the lobe mass becomes engorged with blood and hypertrophies just as when its portal blood supply is increased by obstructing the venous trunk to the main liver;[7] while a sufficient cause for obstruction is to be found in the dilatation under pressure of the main bile

channels of the liver. Betz showed in 1862[13] that a very moderate increase of pressure within the ducts, far less than that developing after total obstruction,[14] can greatly hamper the portal flow. That it does hamper it in the present case, and early, was indicated in one of our animals examined 5 days after ligation of the bile duct to the main liver. The lobe mass was already much engorged and enlarged, although as yet no connective tissue changes had occurred in the main liver that could be invoked to explain a venous obstruction there. The effect on hepatic tissue of the occlusion of portal branches has already been studied by us. There ensues an orderly atrophy with lessening of the biliary activity, and ultimately all parenchyma disappears. Precisely such an atrophy, though one more gradual, results from local biliary stasis; while a diminished biliary activity, at least as regards secretion into the ducts, is indicated by the moderate dilatation of the latter in comparison with their condition when stasis is total, and by the relative rarity of biliary necroses and the slow course of the connective tissue changes.

To obtain additional evidence we have studied the effects of obstructing simultaneously the bile duct and portal branch of a liver mass.

Experiment 1.—Two 1,500 gm. rabbits were operated upon under ether and the bile duct and portal trunk to the main liver twice ligated, just above the caudate lobe. The latter was not taken out. The livers appeared normal at operation, and subsequent examination of the hypertrophic lobe mass showed that cirrhosis had been absent. The animals were killed by bleeding from the heart 5 days and 21 days respectively after operation. They had been in good condition and unjaundiced.

In three other rabbits a mere ligation of the bile ducts to the lobe mass was followed by an occluding thrombosis of the accompanying portal branch. The occlusion must have developed soon after operation, for when the animals were killed the histological findings were identical with those observed in the main liver after simultaneous ligation of both duct and vein.

---

13 Betz, W., *Sitzungsber. k. Akad. Wissensch. Math.-naturw. Cl., Wien.*, 1862, xlvi, 238.

14 Herring, P. T., and Simpson, S., *Proc. Roy. Soc. London, Series B*, 1907, lxxix, 517.

Experimental Unilobular Cirrhosis.

Different stages of the same lesion were found in these rabbits. There was little dilatation of the large bile ducts and only a slight, transient jaundice of the tissue in stasis. A few small parenchymal necroses were seen early, but none later. The lobules rapidly diminished in size by simple atrophy, yet cirrhosis appeared more slowly than when the bile duct alone was obstructed, and, while ringing all the lobules about with a tissue containing many little, new-formed ducts, had not the least invasive tendency. It was, in sum, a pure, evenly distributed, monolobular cirrhosis. Stellate proliferation about the larger ducts was practically absent, as might have been expected from the absence of any marked stretching of their walls. With this exception the picture so entirely resembled that found after occlusion of a bile duct only (Figs. 1 and 2) as to constitute strong evidence for the view that obstruction of the portal stream has much to do with the lesions occurring under such circumstances.

Further Influence of the Portal Obstruction.

The deflection of the portal stream from a liver region in which the bile ducts are dilated as the result of obstruction is probably only partial at first. Later, when cirrhosis develops, it becomes much more nearly complete, as shown by the relatively bloodless condition of the tissue at a period when much parenchyma is still present. Perhaps the arterial circulation is now compromised. Certainly a portion of the blood that comes to the liver passes on to the vena cava through capillaries in the cirrhotic tissue[15] and thus fails of contact with the parenchyma. Pigment secretion into the ducts completely ceases; their dilatation, while still very moderate, comes to a standstill; and the fluid in them becomes colorless and watery. Yet, as our previous work has shown, a main liver mass long deprived of all portal blood, and in advanced atrophy as result, is still capable of forming bile in quantity.[7] The pressure, though, against which secretion takes place into the ducts is greatly diminished, as the following experiment proves.

[15] Ackermann, *Virchows Arch. path. Anat.*, 1880, lxxx, 396.

Experiment 2.—The portal trunk to the main liver mass was ligated in two rabbits of approximately 1,800 gm. weight, and the caudate lobe ablated as usual. 17 and 23 days later, respectively, the bile duct from the mass was ligated just below the entrance of the cystic duct, and 2 and 3 days later the animals were killed and immediately examined. The main liver had atrophied in both instances to less than one-third of the normal weight, while the posterior lobe mass had undergone a corresponding hypertrophy.

In a number of other rabbits the operative procedures were the same, but a gall bladder fistula was made in addition. Certain of the instances in which bile collection was successful have already been described.[7] Sometimes occlusion of the fistula developed, and the animals were killed at various periods thereafter. The findings in such cases deserve consideration with those of Experiment 2 proper, since the conditions are essentially similar.

In these animals with an advanced atrophy of the main liver as result of portal ligation, not the least icteric tinting of the parenchyma or dilatation of the bile passages and gall bladder followed occlusion of the duct; and the passages contained merely a little watery, colorless fluid. Obviously a very slight increase of pressure within the ducts had been sufficient to check secretion into the normal channels. It follows that as hepatic atrophy progresses after local biliary obstruction the chance that lesions will occur through rupture of the ducts, or stasis within them, becomes negligible. Yet when the material for bile formation is provided in unusual quantity a parenchyma deprived of the portal stream can secrete bile into the ligated duct against a considerable pressure, as will now be illustrated by an experiment done with another end in view.

Experiment 3.—A hemolytic and hemagglutinative serum of high titer was obtained by repeatedly injecting a goat with washed, rabbit red cells. A small amount of the serum when given intravenously to rabbits was found to bring about blood destruction over a period of many hours without demonstrable liver injury, whereas large quantities caused necroses. With a properly regulated dosage there was no escape of hemoglobin by way of the urine, even when more than half of the blood of the animal was broken down in the course of only 2 days.

The portal vein and bile duct of the main liver of a 1,700 gm. rabbit were ligated just below the entrance of the cystic duct. 4 days later, when the animal had recovered from the operation and was in good condition, intravenous injections were begun of small doses of the anti-rabbit serum, followed at short intervals by the transfusion of washed, compatible, rabbit red cells suspended in a little salt solution. The amount of serum given was always well below that causing liver changes, but it effected great blood destruction, necessitating five large transfu-

sions to maintain the hemoglobin at the original level. In a period of 11 days 73 cc. of sedimented corpuscles, an amount equivalent to the cell content of 162 cc. of the blood of the animal, or nearly twice the normal total, had been destroyed as the result of but four injections of serum, or 1.7 cc. in all. The animal now possessed a hemoglobin of 86.5 per cent (Palmer) as compared with 82.6 per cent prior to the experiment. It was killed and autopsied. There was at no time any jaundice.

Three rabbits were submitted to a similar experiment for a shorter period of time, and in two others repeated injections were made subcutaneously of large amounts of hemoglobin prepared by the method of Minot and Sellards. No jaundice was observed in any animal.

In these rabbits the bile passages of the main liver and the gall bladder as well were greatly distended and with an abnormally thick, dark green bile. The liver tissue was deeply jaundiced. The changes cannot be contrasted with those in the animals of Experiment 2, since atrophy of the main liver was advanced in the latter before the bile duct was obstructed. They should be compared with the results in Experiment 1, in which a bile duct and portal branch were simultaneously obstructed. Not only was the local accumulation of bile far more copious and the distension of the ducts greater than in the few instances of this experiment, but both were much more pronounced than in any of the rabbits in which, following Nasse's example, we ligated only the duct of the main liver, leaving the portal circulation untouched. It follows that the failure of a liver region deprived of the portal stream to secrete bile against pressure cannot be laid to an essential disability of the cells. In a previous paper evidence has been brought forward that the cells are handicapped in competition with the parenchyma receiving all the portal blood.[7]

Experimental Intralobular Cirrhosis.

A stellate cirrhosis about the larger bile ducts is not rare in human beings as the result of obstruction, with or without infection, and has frequently been brought about experimentally in animals. We have deemed its production in rabbits, apart from other changes, as unnecessary, although logically considered this would be a further step toward separating out the components of the mixed lesion that follows occlusion of the common duct. To obtain, on the other hand,

a type of stasis, and thus presumably of cirrhosis, localized within the lobules, seemed highly desirable. *A priori*, one would expect to meet many difficulties, since it is known that a back pressure throughout the duct system which suffices to cause partial stasis in the lobules almost invariably results in a rupture of some of the bile radicles within Glisson's capsule, and always in connective tissue proliferation about the larger ducts. Our success in obtaining a predominantly intralobular stasis has been the outcome of varied attempts. The condition, and its corollary, a diffuse intralobular cirrhosis, can regularly be brought about in the lobe mass of the liver by diverting the whole portal stream to the latter and ligating the efferent duct. A like result is not produced, we find, when the larger mass of the main liver is subjected to the same procedure, an experiment already performed by Steenhuis.[16] The cirrhosis that then occurs is predominantly extralobular.

Experiment 4.—In eighteen rabbits weighing from 1,500 to 2,400 gm. the bile duct of the posterior lobe—or ducts, for there may be as many as three—was ligated, the portal trunk to the main liver tied off just above the branch to the caudate lobe, and this last ablated as usual. The animals remained in good condition and unjaundiced. They were killed after intervals of from 1 to 33 days.

The operation as described is frequently followed by complications; and the findings in many animals not included in the above number have been ruled from consideration on this account.

The course of the changes has been carefully followed. There is an initial turgor and hypertrophy of the lobe mass resembling that after ablation of the main liver,[17] or simple ligation of the portal branch of the latter,[7] and due of course to the same marked, local increase in the portal stream. Superimposed are destructive changes referable to the bile stasis. The stasis, though brought about by a ligature on the principal duct of the lobe, causes remarkably little dilatation of the latter and still less of its large branches within Glisson's capsule. These are never abnormally prominent on the cut surface of the liver, though microscopically a slight distension and thickening of their walls may be observed after a time, with occasionally some pericholangitis. In the course of several weeks the main duct usually attains a diameter of about 3 mm., that is, becomes twice the normal size, and contains stasis bile, a watery fluid with a few green

[16] Steenhuis, T. S., Experimenteel en Kritisch Onderzoek over de Gevolgen van Poortaderafsluitung, Thesis, Gröningen, 1911.

[17] Ponfick, E., *Virchows Arch. path. Anat.*, 1889, cxviii, 209; 1890, cxix, 193; 1895, cxxxviii, suppl., 81.

solid particles. The brunt of the obstruction falls on the smallest bile radicles in Glisson's capsule, and still more on the canaliculi within the lobules themselves, as shown by the bile thrombi here found and the numerous, minute parenchymal necroses. Such necroses appear within a few hours after the ligation and new ones develop day by day. Usually they cannot be seen with the naked eye, involving, as they most often do, one to three or four cells scattered throughout the lobule from periphery to center. The dead cells stand forth prominently in the liver cords, staining a bright pink with eosin, and lacking nuclei (Fig. 3). By the 3rd day they are numerous, especially in the outer and mid-zone of the lobule, and occasionally a few larger necroses involving ten or fifteen cells may now be present. Some of the latter are undoubtedly the *Netz-Nekroses* which Steenhuis[16] saw in liver tissue hypertrophic as result of an increased portal flow, but others have the character of frank biliary necroses. Very exceptionally they involve considerable lobular segments and may then attract the unaided eye. In animals showing them the smaller, disseminated necroses are especially numerous. It is the latter that characterize the lesion and continue steadily to be formed. Being purely parenchymal they are rapidly replaced by proliferation of the lobular cords without connective tissue participation, and their number is never sufficient at any one time to render them confluent. They appear to have no relation to the small, scattered, intralobular bile thrombi that, during the first few days, and then only, are numerous enough to attract attention.

The severity of the lesion varies largely with the individual. By the 3rd day in some cases, or the 6th or 7th in others, a new element makes its appearance; namely, a proliferation in the finer ramifications of Glisson's capsule. Fibroblasts begin to penetrate within the enlarging lobules (Fig. 4), and by the 12th day as a rule many thin strands of them can be found, so far toward the central vein and so isolated in cross-section that were the absence of connective tissue within the normal parenchyma not well attested[18] one would conclude that they had developed *in situ*. New-formed bile ducts have now made their appearance in the interlobular connective tissue which is definitely increased in amount.

The lobe mass at this time is highly interesting in the gross. It is enlarged, sometimes to double its normal size, with rounded contours and blunt edges. The bulging hypertrophic lobules render the surface slightly and regularly uneven. The tissue is firm and somewhat inelastic, and the knife meets a smooth, non-crepitant resistance. The color is gray-pink, in contrast to the purple of the main liver. When the vessels are severed nearly all the blood is forced out by the turgid tissue, and the general tint is then a uniform, pale buff, or putty color. On the surface laid open the lobules can with difficulty be made out, slightly raised, in a matrix of gray, translucent, connective tissue which appears to penetrate and overgrow them. Sometimes their position can be made out only through the situation of the gaping central veins. There is now no definite jaundice of the tissue, though its yellowish tint is suggestive. Earlier, during the

[18] Mall, F. P., *Am. J. Anat.*, 1906, v, 227.

first few days after the ligation, the enlarged lobe may be a greenish red-purple, though jaundice of the animal never occurs.

By the 21st day the lobe mass, still in process of hypertrophy, contains nearly as much connective tissue as parenchyma. Almost everywhere fibroblasts have penetrated to the central veins of the lobules, though so regularly that the original pattern of the liver is still fairly maintained. The cell cords, though, are distorted and much fewer. The necrosis of individual cells and small cell groups is still going on, as is also proliferation of the surviving parenchymal elements which individually may be much above normal size. The interlobular connective tissue is greatly increased, both relatively and actually, and everywhere throughout it are new-formed bile ducts. About some of the small hepatic veins is a collar of young connective tissue containing in its midst irregular strands of small, compressed looking cells, superficially suggestive of new-formed bile ducts but in situation widely separate from the latter and with tinctorial differences that identify them as parenchymal.

At this time the lobe mass is of a pale flesh color and appears almost bloodless compared with the main liver (Fig. 9). In truth it is so, owing to interference with the portal stream by the cirrhotic tissue, which usually begins before the 12th day and has now become marked. Venous collaterals open, sometimes as a *caput Medusæ*, when the omentum is attached by adhesions to the old wound, more usually as small, direct anastomoses between the portal branches and vena cava, and oftenest as dilated Charpy's veins along the gastrohepatic omentum to the main liver. But all of the new channels fail as a rule to avert a serious chronic passive congestion. As result the spleen is changed into a tense cylinder (Fig. 9) and may weigh more than 4 gm., nearly six times the average for normal rabbits of similar size. Occasionally ascites occurs. On cutting into the lobe mass its large, portal channels are found to end bluntly in small veins.

The surface of the lobe is still finely rugose, and its substance is now extremely resistant to the knife, though non-crepitant. When laid open the glistening, bloodless, pinkish gray tissue shows no distinct pattern of lobules, but these can usually be made out when viewed obliquely, since they are slightly raised above the general surface. Some appear larger than normal whereas others blend with the connective tissue. The main liver mass fails to undergo the atrophy which in the lack of a functional need would be its fate.[7]

By the 30th day cirrhosis has progressed much further and so has the development of collaterals which shunt the portal blood around the lobe mass. The adequacy of the new channels is now shown by the disappearance of all signs of passive congestion. The lobe mass has in consequence come to lie outside of the venous stream, being reduced to much the same circulatory condition as if its portal branch had recently been tied. Hypertrophy ceases, and atrophy takes its place. But though the lobe mass is still much above normal size little parenchyma is left to undergo retrogression. The mass consists almost entirely of connective tissue with an abundance of new-formed bile ducts and some round cells. Here

and there are to be found small islands of liver cords, and separated cords and cells (Fig. 5), some of them dying, though whether as a result of the bile stasis or of the cirrhosis we have not sought to discover. The liver destruction has far surpassed that compatible with life were the whole organ affected. Beyond the 33rd day the changes have not been traced.

Altogether the type of cirrhosis is as purely intralobular as is compatible with the fact that the lobules normally contain no connective tissue,[18] so that its appearance and proliferation within them must of necessity take place by penetration from without.

Influence of an Increased Portal Stream.

The secretory activities of a lobe mass receiving the entire portal stream but with bile duct ligated, as in the preceding experiment, become strikingly evident when the duct from the main liver is also tied with result in total obstruction. There is now no relief for the lobe mass such as might be afforded through the efforts of a parenchyma with unobstructed bile outlet.

Experiment 5.—In ten rabbits of 1,500 to 2,200 gm. an operation similar to that of Experiment 4 was successfully performed and in addition the bile duct from the main liver mass was ligated. In eight cases the gall bladder was removed in order to render the conditions in the main liver more strictly comparable with those in the lobe mass. The animals died, or were killed, after periods of 1 to 12 days. Cultures taken from such as died showed that the liver lesions were not referable to infection; and as further proof the histological findings agreed in all ways with those of animals killed while still in good condition.

The animals of this experiment were more seriously affected than if total biliary stasis had been produced in the ordinary way by obstruction of the common duct. A sufficient explanation is to be found in the radical circulatory derangement.

Only two animals survived for as long as 12 days. Jaundice always developed within 48 hours, and rapid emaciation was the rule; yet despite the poor general condition the lobe mass of the liver underwent some hypertrophy. Its turgid tissue became brightly and evenly bile-stained—green, or ocher-colored. The surface remained smooth, and at no time were biliary necroses perceptible in the gross (Fig. 10). The lobules were definitely enlarged and appeared regular, save that near the central vein there was some opacity with a brilliant bile tinting. The impression here gained was not that of a diffuse necrosis but of intense im-

pregnation of living tissue with bile pigment. This was the actual state of affairs, as sections showed. Toward the center of the lobules pigment existed in great quantity as small bile thrombi (Eppinger), as large, rounded, brownish black masses (Fig. 6), intracellular particles, and as a diffuse stain for groups of dead parenchymal cells. No such marked condition has to our knowledge been encountered heretofore, though for that matter the factors in its production can scarcely be expected to come together naturally.

The earliest histological changes were like those observed in the companion Experiment 4, in which the duct of the main liver was left open. There was the same scattered necrosis of individual cells throughout the lobule, but in much more pronounced form (Fig. 7), and dilatation of the ducts was greater. Small concretion-like pigment "thrombi" appeared early here and there between the parenchymal cells, and after but a few days the pigmentary accretion was, as already described, enormous (Fig. 6). The number of dead cells grew rapidly, and close to the center of the lobules larger biliary necroses occurred, some deeply impregnated with bile, but none of such size as to attract the unaided eye. These features acted to modify the rapidly developing cirrhosis, which in the animals surviving longest was intralobular both by diffuse invasion and by the replacement of necroses. Pericholangitis of the larger ducts was absent. At the 12th day active division of the surviving parenchymal cells was still going on; some elements were of greatly increased size, and nuclear irregularities were frequent.

The main liver showed only the lesions that ordinarily follow obstruction of the common duct, combined with the slight atrophy inevitable to deprivation of the portal stream. The local accumulation of pigment was slight, as compared with that in the posterior lobe mass, yet large biliary infarcts were numerous (Fig. 8).

The extraordinary impregnation with pigment of the posterior lobe mass (Figs. 6 and 7) contrasts strikingly with the slight jaundice of the main liver (Figs. 8 and 10). Since biliary activity is dependent, in large part at least, upon portal flow, and this is greatly different in the two liver portions, such a contrast need not occasion surprise. The absence of gross necroses, though, in the posterior lobe mass is difficult to reconcile with the pronounced biliary leakage there occurring save on the assumption that the increased portal flow provided a circulation to cells that otherwise would have been deprived of it by the extravasations.

The Factor of Safety in Bile Elimination.

The tissue of the lobe mass in Experiment 4 was jaundiced only during the first few days after operation. Experiment 5 clearly shows that bile pigment must have been prevented from accumulating here,

as well as in the organism generally, through the activities of the main liver. When there exists only an uncomplicated biliary obstruction of the main liver, the posterior lobe mass functions in the same way to prevent a general jaundice, as has already been noted. It is remarkable that the literature contains no direct recognition of the fact that a small portion of the liver may serve for the whole as far as regards the elimination of bile pigment. On the contrary, one finds everywhere the statement that the severity of jaundice is directly proportional to the amount of liver tissue in stasis; and small, local lesions have been invoked to explain its clinical occurrence. The subject merits a separate paper and will be considered here only in so far as it affects our theme. But this it does to a considerable degree.

The factor of safety in bile elimination is strikingly illustrated by some recent experiments for another purpose performed by one of us and Dr. Philip D. McMaster. The bile duct from the main liver was tied, and so too was the portal branch to the lobe mass from which the caudate lobe was ablated as usual. Owing to the increased portal stream to the main liver the tissue of the latter must be supposed to have formed bile in unusual quantity, and this found no outlet by the normal route. Yet the eight rabbits of the series remained in excellent condition and unjaundiced, although the bile could be eliminated only through the lobe mass, less than a quarter of the total substance of the liver, and supplied with blood only through the hepatic artery, which, judging from observations on dogs,[19] provided only about 40 per cent of the normal blood supply.

Local Effects of Increased Bile Secretion.

It has repeatedly been suggested on the basis of clinical evidence that increased secretion, or the secretion of an abnormally thick bile, may lead to stasis within the ducts and thus injure the liver eventually. Hanot's cirrhosis was at one time attributed to such cause. But certainly small portions of the liver of the rabbit can rid the body of large amounts of the animal's highly irritant bile without suffering injury. This was true in all of the instances of compensatory secre-

[19] Macleod, J. J. R., and Pearce, R. G., *Am. J. Physiol.*, 1914, xxxv, 87.

tion described in the present paper. Even when the bile is thickened as the result of hemolysis and the whole brunt of its elimination is abruptly thrown upon the lobe mass of the liver, the latter suffers no damage visible histologically. Experiment 3 was originally devised to test this point.

DISCUSSION.

Physiological Pathology of the Lesions.

Rabbit bile that has escaped from the ducts is highly injurious to the tissues, causing necrosis even of the liver parenchyma.[20] In this fact and in the differing paths by which the fluid may leave the collecting tract under various conditions of obstruction are to be found primary reasons for the diverse types of biliary cirrhosis which we have produced experimentally.

The cirrhosis which follows ligation of the common duct is, as has already been pointed out, a mixed lesion. So considerable is bile secretion despite the stasis, that no part of the collecting tract from the intralobular canaliculi to the main duct, inclusive, escapes the effects of the irritant fluid. Pressure changes also occur, and the local condition is probably aggravated by the resecretion of biliary constituents from the heavily charged blood,[21] which would greatly favor accumulation within the hepatic tissue. Though most of the secretion must continue to find its way back into the blood and lymph, the process cannot but be faulty when the body fluids are already heavily laden.

The progressive atrophy and orderly cirrhosis that result from obstruction of a single large bile duct are traceable to the interaction of two opposing sets of factors, one set vicious, the other tending to relieve the conditions. The initial bile stasis leads to rupture of some of the finer ducts with parenchymal necrosis, and its continuance to an interlobular and pericholangitic connective tissue growth. Probably this cirrhosis interferes eventually with the general blood

[20] Bunting, C. H., and Brown, W. H., *J. Exp. Med.*, 1911, xiv, 445.

[21] Bile constituents injected into the circulation are rapidly taken out by the normal liver—*vide* Stadelmann, E., Der Icterus, und seine verschiedenen Formen Stuttgart, 1891.

supply. There is no doubt that the portal flow is partially diverted from the beginning through the encroachment of distended bile ducts on the stream bed, and hence there ensues a gradual atrophy of the parenchyma such as follows any local portal deprivation.[7] But while destruction is thus being effected through a combination of several influences others act to limit the injury. The liver portion with duct still open keeps the organism more or less free from bile and thus tends to prevent resecretion into the region of stasis; secretion is still further reduced by the diverted portal flow;[7] and the direct effects of such stasis as nevertheless ensues are lessened by the passage of biliary constituents from the obstructed tissue into a blood and lymph relatively free from them. As parenchymal atrophy advances the pressure under which bile is secreted into the ducts decreases greatly (Experiment 2), and in consequence their rupture, with extravasation, no longer occurs. Furthermore, with the passing of time the functional responsibilities of the tissue in stasis decrease, owing to hypertrophy of the unobstructed liver mass. And so through several means the local changes are rendered mild and orderly. Similar lesions from similar causes, but with less of cirrhosis and a more rapid atrophy, as would naturally follow from the circumstances of the case, are seen when a bile duct and its corresponding portal branch are occluded at the same time (Experiment 1).

The predominantly monolobular cirrhosis which follows the occlusion of a single duct, and the purely monolobular lesion that develops when the corresponding portal branch is also tied, are referable not to rupture of the ducts, which is negligible, but to continued passage through their walls of the irritant bile. When a colored solution is introduced into the bile passages under a pressure that fails to rupture them it escapes with ease through the walls of the finest radicles in Glisson's capsule.[18] And precisely here, as indicated by the connective tissue lesion, does the bile pass into the tissues under the circumstances we are now discussing. This path of egress would seem sufficient to relieve the stasis when secretion has been cut down by the ligation of the portal branch to the affected region, for under such circumstances lesions are not observed elsewhere in the liver. When the duct alone has been ligated secretion and stasis are both greater, and in addition to the monolobular cirrhosis a stellate proliferation is then found about the large, distended ducts.

No clear reason is evident for the intralobular situation of the stasis that results from obstructing a single bile duct and greatly increasing the local portal flow. The changes that ensue, though, are easily understood. An increased portal flow of itself fails to induce connective tissue changes.[17] They may be attributed in the present instance to the numerous bile leaks within the lobules, leaks which also cause a profusion of punctate necroses. The lymph from the affected tissue comes away more or less laden with bile. It passes, not direct from the lobules into the formed lymphatics of Glisson's capsule, but first through unlined spaces between the connective tissue cells.[18] Here its biliary component has abundant opportunity to cause irritation, and here consequently at the edge of the lobules connective tissue proliferation occurs, and fibroblasts penetrate rapidly between the liver cords, following up, so to speak, the path of the irritant fluid. A diffusely intralobular cirrhosis is the result. The absence of large biliary infarcts, despite the great secretory activity of the tissue (Experiment 5), may be due in part to the support given the walls of the bile radicles by the increased portal pressure, which would also act to maintain the blood current despite local extravasations so that the latter affect relatively few cells. The observation has been made (Experiment 5) that when there is an increased portal flow in one part of the liver, an absence of such flow in the remainder, and biliary obstruction in both, gross biliary necroses fail to occur in the first mentioned liver portion, although the jaundice there is extreme, whereas in the second portion, which is unjaundiced, and must be relatively inactive, large infarct-like necroses appear (Fig. 8). To judge from this there would appear to be much in the current belief that biliary necroses, though caused in some part by the direct injury of cells by extravasated bile, owe their size when large to interruption of the blood current.

Interpretation of Human Lesions.

Many facts warrant the application of the principles here set forth to the frank biliary lesions of human beings. The essential likeness of the changes in man and the rabbit has been briefly indicated in an earlier part of this paper. As Quincke and Hoppe-Seyler put the

matter, summing up their more extensive comparison: "From all this it would seem that the findings in guinea pigs and rabbits are analogous in many ways to those in human beings."[5] But it is in essentials only that the findings are analogous. The relatively innocuous character of human bile, the slow rate at which it is secreted, the greater frequency of infection in areas of stasis, all act to vary the picture. Furthermore, the liver parenchyma may, like that of the dog, respond rather slowly to changed circulatory conditions[7] such as bile stasis induces. Certainly the intralobular bile canaliculi are, like those of the dog and cat, relatively inaccessible to pressure exerted through the large ducts,[18] so that they are only slightly implicated by stasis in these latter.

The forms of cirrhosis associated with Hanot's name cannot on present evidence be classed as of frank biliary origin. Nevertheless, our observations bring something of clarity into a consideration of them. Whether there exists an entity of the sort described by Hanot has been much debated, and there is great discordance in its definition by recognized authorities. No one disputes, on the other hand, the occurrence in man of a peculiar, progressive, hepatic cirrhosis, accompanied by more or less jaundice, and resulting usually in an enlarged liver in which are found inflammatory lesions of the smaller bile ducts with signs of stasis in them. It is our belief that the diversity of the liver changes is referable to differences in the duct levels at which the injurious agent is active, differences which in the rabbit lead, as we have shown, to cirrhoses of strikingly individual character. In rabbits, and with bile as the irritant, a localization to the intralobular bile canaliculi leads to a diffuse intralobular cirrhosis, whereas when the smallest bile radicles in Glisson's capsule are affected there results a pure monolobular cirrhosis, and implication of the large bile channels leads to a stellate proliferation about them. Parallel instances, pure and mixed, of all except the last mentioned lesion, are to be found within the group of the hypertrophic biliary cirrhoses of man. The rapidly progressive type of the disease, frequent in Indian children, is almost entirely of intralobular character, as Gibbons' excellent description shows.[22] There

[22] Gibbons, J. B., *Sc. Mem. Med. Off. India*, 1891, vi, 51.

is here a marked degeneration of the liver cells. According to Kaufmann,[23] with whom relatively few agree, intralobular growth is a distinctive character of Hanot's disease. In Lereboullet's monograph[24] most of the instances are monolobular, with occasionally an intralobular and sometimes a perilobular involvement.

The hypertrophy of the surviving parenchyma in Hanot's cirrhosis is not more difficult to understand than that usual to atrophic cirrhosis and here regenerative in character.[25] In explanation of the continued connective tissue growth which renders the liver large, one need only invoke the constant stimulus to proliferation that is undoubtedly present, as the angiocholitic lesions show. By contrast, injury is intermittent in the atrophic cirrhosis of Laennec, and at death, when a contracted scarring is found, often no signs of recent damage can be made out. The round cell aggregations present in hypertrophic biliary cirrhosis need no other cause than the chronicity of the inflammation. And the not infrequent chronic passive congestion occurring at a late stage with or without a marked diminution of the size of the liver, is referable to secondary connective tissue shrinkage and to the localization of the cirrhosis in the individual case. In our rabbits with hypertrophic biliary cirrhosis chronic passive congestion regularly developed as a late feature.

The ability of the least harmful bile of which the effects have been carefully followed, that of the cat, to elicit eventually a liver cirrhosis in the absence of infection[6] might well lead one to stress this factor as a cause of connective tissue changes in the human liver. The occurrence of intense local bile-staining without such lesions, a phenomenon not infrequent at autopsy in liver diseases, carries no weight in this connection, since the chronicity of the local stasis is here unattested. But Hanot's cirrhosis has certainly a varied etiology. The infantile form so frequent in India probably has its own specific cause. A number of well known microorganisms have been isolated from the liver in occidental forms of the disease, though the readiness with which infection supervenes on bile stagnation leads one to doubt

[23] Kaufmann, E., Lehrbuch der speziellen pathologischen Anatomie, Berlin, 1907.

[24] Lereboullet, P., Les cirrhoses biliaires, Thèse de Paris, 1902.

[25] Kretz, R., *Wien. klin. Woch.*, 1900, xiii, 271.

that their rôle is primary. Hanot's early hypothesis of a primary bile stagnation as the result of a "biliary diathesis" seems to us worthy of reconsideration in certain cases. The essential chronicity of the changes accords well with the relatively innocuous character of human bile; while the enlargement of the spleen which is sometimes great, and the occasional familial character of the malady are facts suggesting an inherent peculiarity of the patient closely akin to that of congenital familial jaundice, as several authors have not failed to point out. Yet it should be remarked in this connection that cirrhosis is not a feature of the latter disease, though for years the liver secretes a tenacious, heavily pigmented bile. Furthermore, we have been unable to cause liver injury in the rabbit by many times increasing the bile output of pigment from small portions of parenchyma (Experiment 3).

The possibility should be borne in mind that local bile stasis may act to complicate any chronic liver derangement in which bile passages, small or large, are compromised. That obstruction to the smaller channels may be responsible for certain of the supposedly "unobstructive" jaundices has been proved by Eppinger. Yet the absence of clinical jaundice is, as we have found, compatible with a condition of total obstruction in more than three-fourths of the liver of the rabbit. Should the same hold true for the human liver the existence of a "*cirrhosis biliaire anicterique*" such as has been described by French authors will not be difficult to understand.

SUMMARY.

There are excellent reasons for employing the rabbit in an experimental analysis of the biliary factor in liver lesions; and it is possible to obtain in this animal results uncomplicated by infection or by intercurrent cirrhosis.

Ligation of the common duct of the rabbit results in a mixed lesion from injury throughout the entire length of the bile channels. By obstructing single ducts and altering the portal stream we have produced cirrhoses of pure monolobular and diffusely intralobular types. The character of the connective tissue changes is determined by the path of escape of bile from the collecting system, which in turn is

largely conditional upon the secretory activity, while this again is dependent upon blood flow. The portal flow is largely diverted from regions of local stasis through encroachment on the stream bed by the dilated ducts.

There is a large margin of safety in bile elimination by the normal hepatic tissue. Less than a quarter of the liver of the rabbit, and this deprived of its entire portal stream, will suffice to keep the organism free from clinical jaundice and healthy when the remainder of the liver, which receives all of the portal blood, has its ducts ligated. The vicarious elimination thus illustrated is of great importance for regions of local stasis by keeping the blood relatively free from bile, thus preventing resecretion into such regions and facilitating exchange from them into the body fluids.

Our experimental monolobular and intralobular cirrhoses are the result of the limitation of biliary lesions to special levels of the duct system. Their resemblance to the different forms of "biliary" cirrhosis associated with Hanot's name is close, and the diverse liver lesions of Hanot's disease are readily explained on the assumption that the stasis, with or without infection, which is indubitably here present, has its situation at different levels in different cases. There are reasons for the view that bile stasis *per se* may sometimes be a prime cause of the malady. Certainly such stasis must be thought of as acting to complicate many chronic liver lesions.

In a later paper experiments on the dog will be described essentially similar in result to those on the rabbit as here set forth.

EXPLANATION OF PLATES.

Plate 17.

Figs. 1 and 2. The lesions after 14 days occlusion of the bile duct from the main liver mass (Fig. 1), and after 22 days of such occlusion plus ligation of the corresponding portal trunk (Fig. 2). There is an identical interlobular cirrhosis in both cases with many new-formed bile ducts, simple atrophy of the parenchyma, and a dwindling in size of the lobules. The scattered dark spots are pigmented Kupffer cells such as are found after any local portal obstruction. Hematoxylin and eosin.

Plate 18.

Fig. 3. Condition of the lobe mass of the liver 2 days after the operation to produce hypertrophic cirrhosis. Necrotic parenchymal cells can be seen here and there. Two are indicated by arrows. The lacunæ near the center of the

lobule on the left indicate where bile thrombi have been dissolved out by the fixative. Eosin and methylene blue.

Fig. 4. Experimental hypertrophic cirrhosis after 13 days. In the center of the field is an unusually well preserved lobule with fibroblasts penetrating it from all sides. Elsewhere only irregular cell cords can be distinguished amid the cirrhotic tissue. Hematoxylin and Van Gieson's stain.

Plate 19.

Fig. 5. Experimental hypertrophic cirrhosis after 30 days. The parenchyma is almost entirely replaced by connective tissue containing many new-formed bile ducts. The position of the lobules can no longer be made out save from the central veins. One of the latter is here shown with some degenerating liver cords near it, while elsewhere a few parenchymal cells can be seen. Eosin and methylene blue.

Fig. 6. Pigment accumulation in the lobe mass 7 days after diversion to it of the entire portal stream and ligation of the common duct. Masses of bilirubin are to be seen, especially near the center of the lobules. There is a beginning cirrhosis. Fresh specimen stained with methylene blue.

Plate 20.

Figs. 7 and 8. The lesions 7 days after diversion of the whole portal stream to the lobe mass and ligation of the common duct. In Fig. 7, of the lobe mass, one finds a punctate parenchymal necrosis which is far more pronounced than when biliary obstruction is local (Fig. 3), while the number of bile thrombi, as indicated by lacunæ, is much greater. In the main liver (Fig. 8) necrosis takes a gross form although the other signs of stasis are slight. Both specimens have the same magnification. The cells of the lobe mass are relatively very large. Eosin and methylene blue.

Plate 21.

Fig. 9. Experimental hypertrophic cirrhosis after 21 days. The fleshy, pink mass with slightly roughened surface next to the pylorus is the cirrhotic lobe mass, well nigh bloodless owing to the obstruction of its vessels. The spleen, in chronic passive congestion, is a much enlarged, tense cylinder, despite the collateral vein running from it to the main liver. There was marked ascites in this instance.

Plate 22.

Fig. 10. Results of 7 days diversion of the portal stream to the lobe mass of the liver with ligation of the common duct. The lobe mass is enlarged and deeply jaundiced, but without evident necroses, whereas the main liver, somewhat smaller than normal and almost unjaundiced, shows large biliary infarcts.

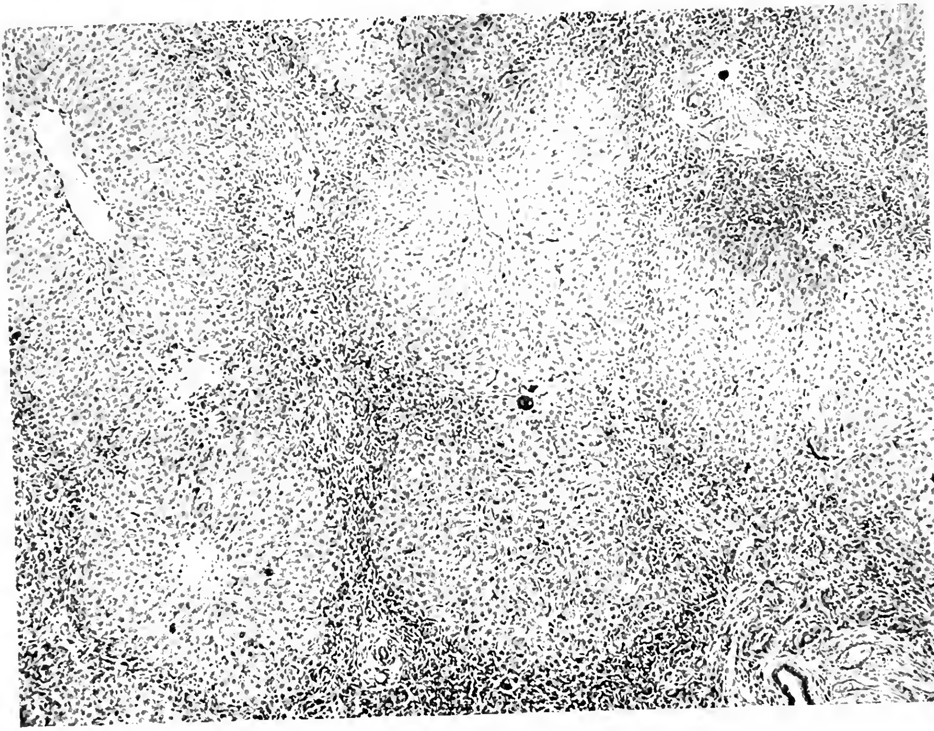


FIG. 1.

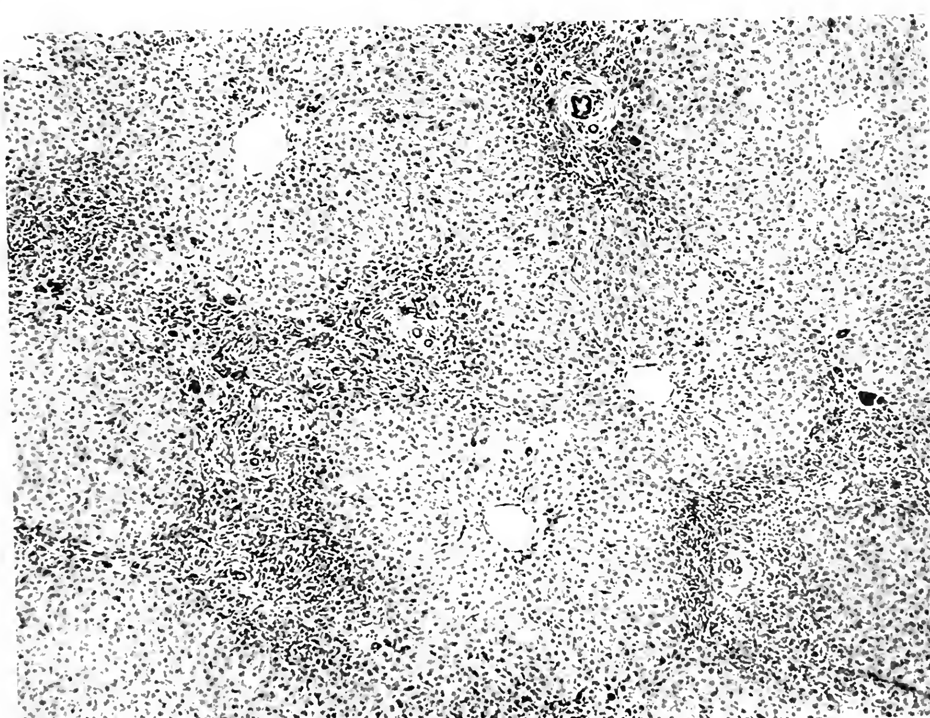


FIG. 2.

(Rous and Larimore: The biliary factor in liver lesions.)

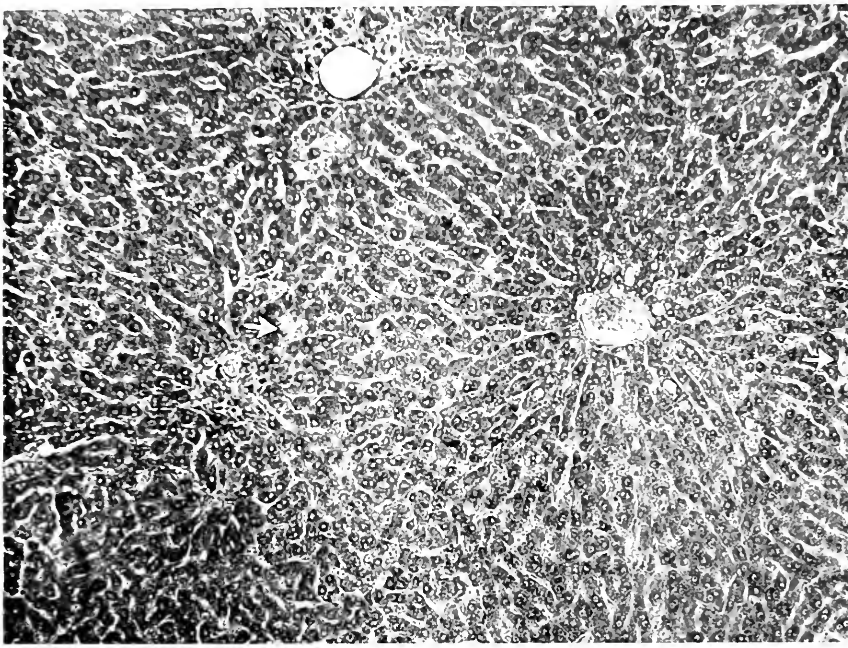


FIG. 3.

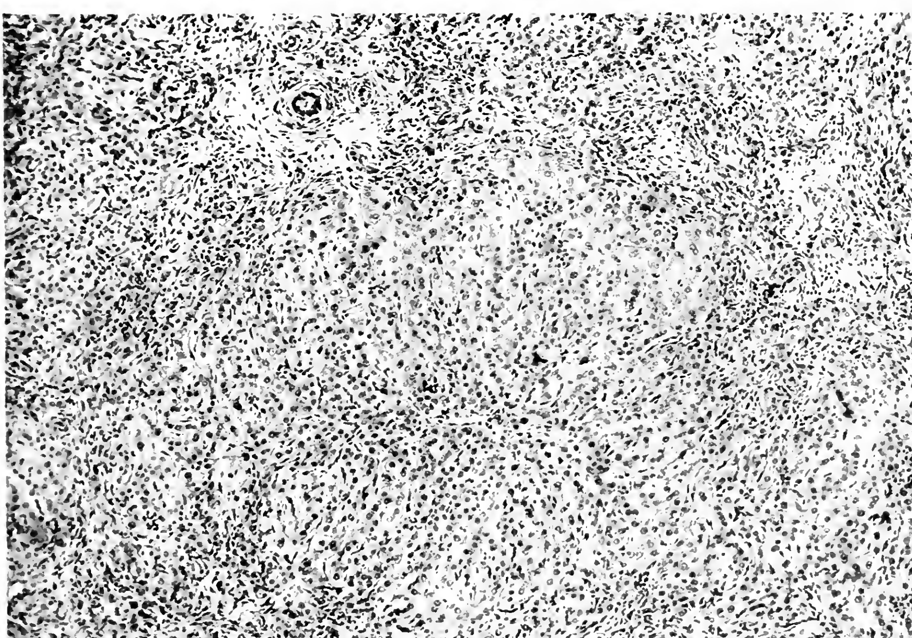


FIG. 4.

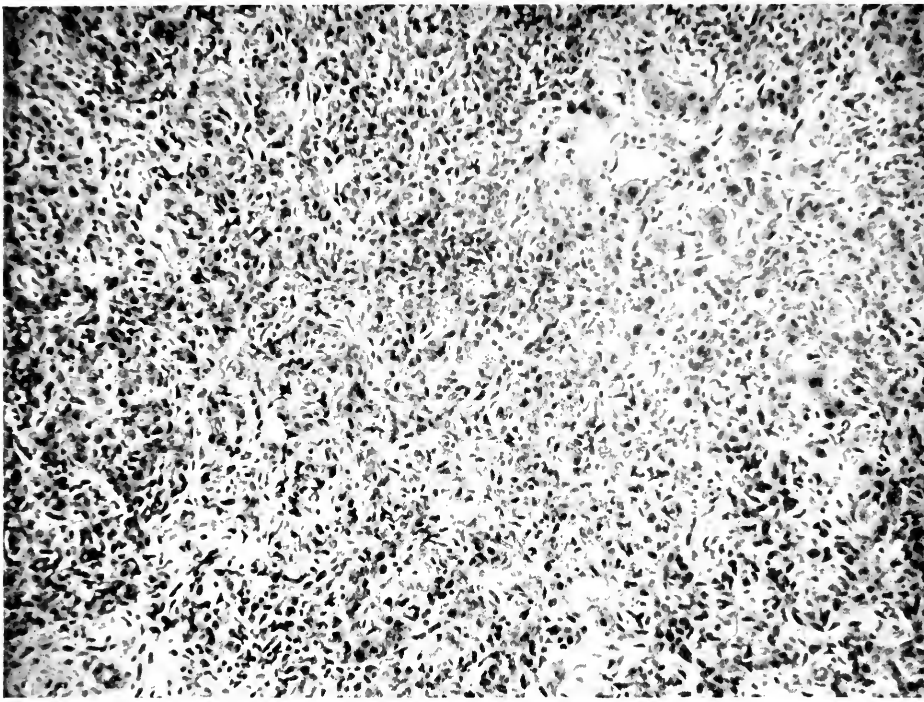


Fig. 5.

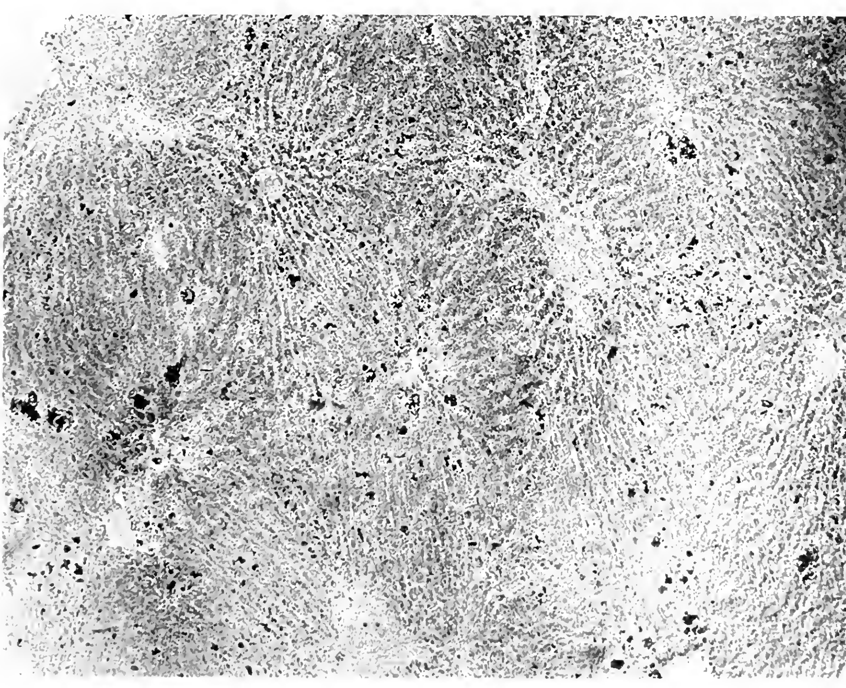


Fig. 6.

Rous and Larimore: The biliary factor in liver lesions.

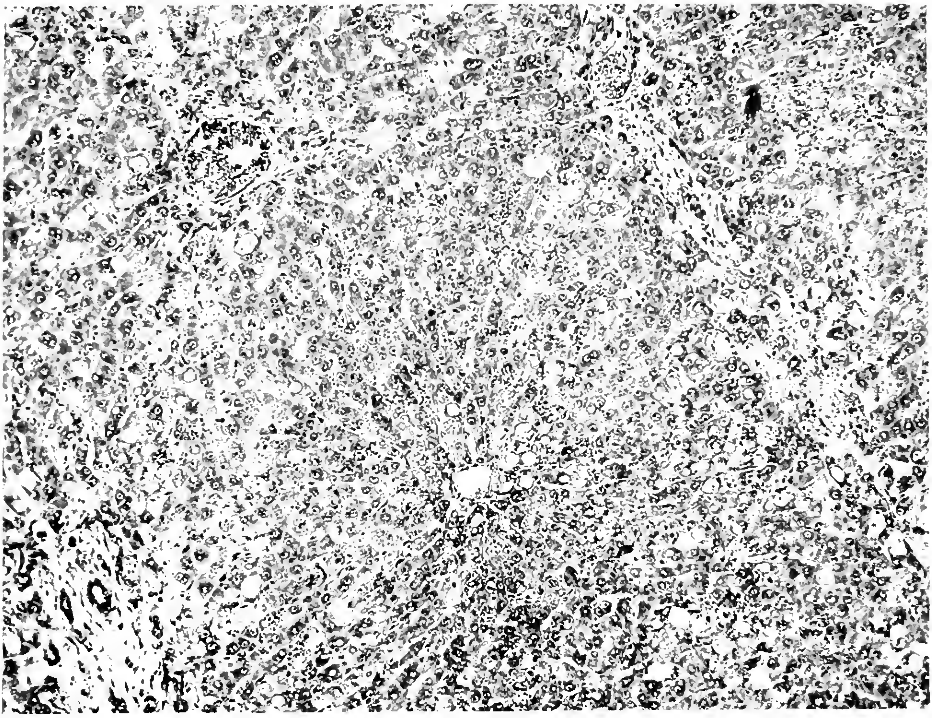


FIG. 7.

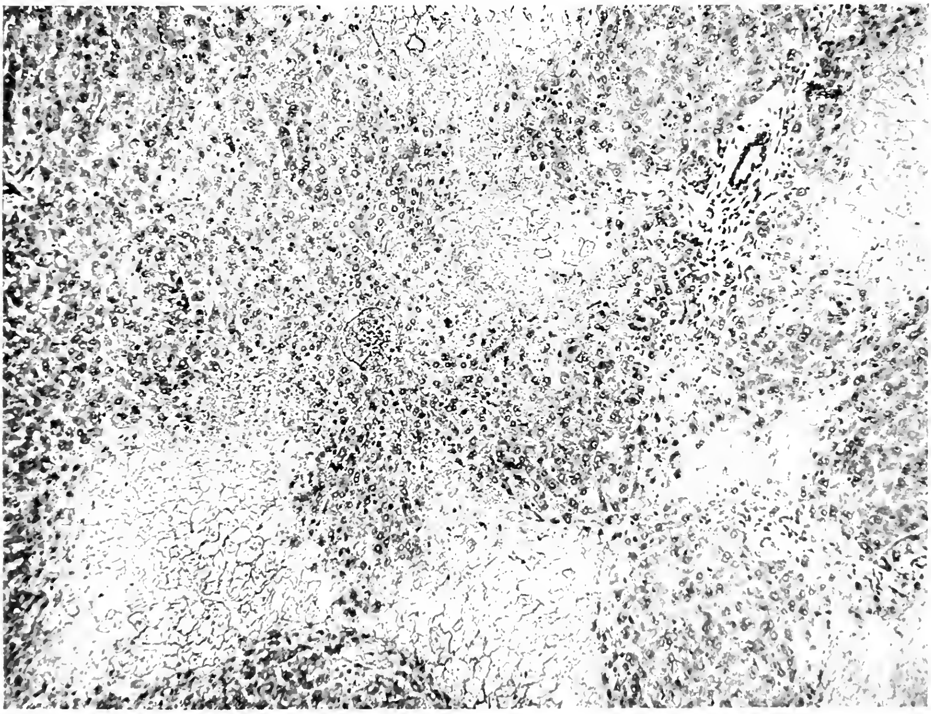


FIG. 8.

(Rous and Larimore: The biliary factor in liver lesions.)

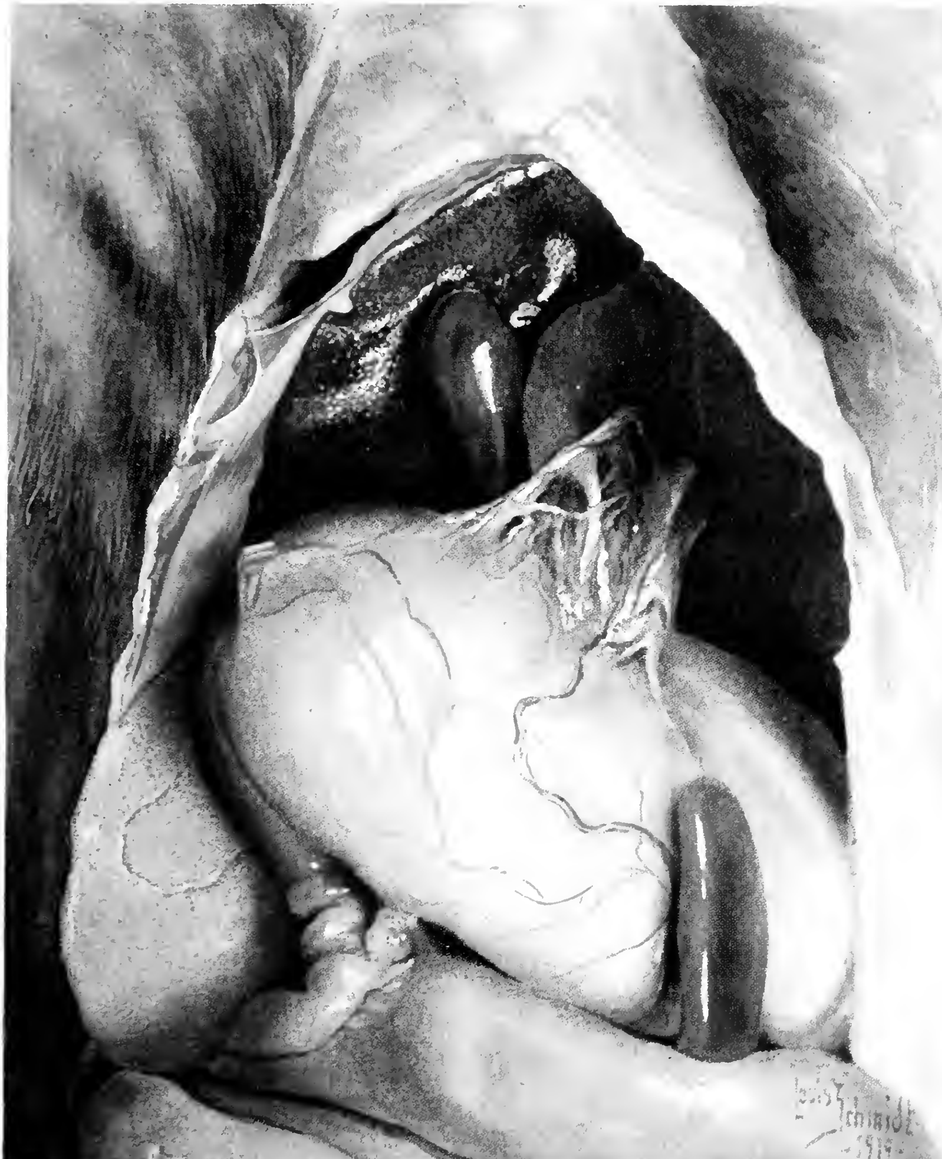


FIG. 9.

(Rous and Larimore: The biliary factor in liver lesions.)

INFLUENCE OF VARIATIONS OF MEDIA ON ACID PRODUCTION BY STREPTOCOCCI.

By F. S. JONES, V.M.D.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

(Received for publication, May 5, 1920.)

In an attempt to differentiate species of organisms closely resembling each other, the fermentation of carbohydrates and other substances plays an important part. When the fermentation characters of streptococci were first studied indicators such as litmus were employed which revealed only qualitative changes. Later quantitative determinations of acid by titration against solutions of alkali came into use.

More recently Clark and Lubs[1] have employed the hydrogen ion concentration method as an aid in the differentiation of closely allied species of organisms. Several have applied it for determination of acid production by streptococci. The advocates of the newer method point out that it is more accurate than titration since it indicates only free acids. The term "limiting or final hydrogen ion concentration" has come into use, since it indicates the maximum acidity or alkalinity produced by a given organism. Thus Avery and Cullen[2] have shown that human streptococci grown in veal infusion bouillon containing 1 per cent dextrose attained a limiting hydrogen ion concentration of 5.0 to 5.2. The bovine group produced more acid, 4.3 to 4.5. Ayers, Johnson, and Davis[3] some time previously had grown human hemolytic streptococci in a desiccated yeast-peptone-dextrose medium, one part of each per 100 parts of water, and found that the bulk of their strains reached a final hydrogen ion concentration of 5.4 to 6.0; 5.6 seems to have been the average. Smillie,[4] who used 1 per cent dextrose in fermented veal infusion bouillon, records the figures pH 5.1 to 5.4 for a few human strains. Brown[5] observed a limiting acidity of pH 5.1 to 5.4 for human streptococci grown in plain bouillon containing 1 per cent dextrose.

---

[1] Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 1.

[2] Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1919, xxix, 215.

[3] Ayers, S. H., Johnson, W. T., and Davis, B. J., *J. Infect. Dis.*, 1918, xxiii, 290.

[4] Smillie, W. G., *J. Infect. Dis.*, 1917, xx, 45.

[5] Brown, J. H., *J. Exp. Med.*, 1920, xxxi, 35.

Fennel and Fisher[6] record the acid limit of *Streptococcus hæmolyticus* as pH 4.5. Whether these figures include both the human and bovine varieties is not stated.

Since considerable variation in acid production had been observed by various workers it seemed possible that the differences might be the result of variations in the media. Broadhurst[7] brought out the difference in the amount of titratable acid produced by streptococci grown in broth prepared from meat and meat extract. Streptococci grown in the former media produced two or three times as much acid as those grown in meat extract media. Many have observed the marked increase in the amount of titratable acid when more than 1 per cent of peptone was used in sugar broths. The writer has frequently noted that streptococci grown in fermented bouillon containing 1 per cent dextrose to which sterile serum had been added invariably produced more titratable acid than cultures in the same media which did not contain serum. A small number of tests also showed that the hydrogen ion concentration was often greater in the dextrose serum broth. The results, then, were not readily explicable on the assumption that the buffer activity of the serum was responsible for the increase in the acidity. It seemed desirable to note the effect of variations of the medium upon acid production.

Veal infusion was prepared from the flesh of a calf 1 day old. It is customary in this laboratory to add two parts of water to one part of the ground flesh. The infusion was divided. To one portion 1 per cent of peptone (Fairchild's) and 0.5 per cent of sodium chloride were added and the reaction was adjusted to + 0.8 (pH 7.6). The remainder of the infusion was strained and inoculated with a young culture of *Bacillus coli* and permitted to ferment at 38° C. for 18 hours. After straining through cotton and gauze, the usual quantity of peptone and salt was added to the larger portion of the filtrate. The reaction was adjusted to + 0.8 (pH 7.6). To a smaller portion of the fermented infusion 2 per cent of peptone and the usual amount of sodium chloride were added. This then provided three types of bouillon prepared from the same materials, unfermented broth and fermented bouillon containing 1 and 2 per cent of peptone respec-

[6] Fennel, E. A., and Fisher, M. B., *J. Infect. Dis.*, 1919, xxv, 444.

[7] Broadhurst, J., *J. Infect. Dis.*, 1913, xiii, 404.

tively. The broths were tubed in amounts of 13 cc. After sterilization under pressure, 1 cc. of a sterile 13 per cent solution of dextrose was added to each. The columns of liquid in the tubes ranged from 6 to 6.5 cm. in height. To half of the tubes of fermented and half of the tubes of unfermented bouillon, 0.5 cc. of sterile horse serum per tube was added. The 2 per cent peptone-dextrose fermented broth was used without other additions.

All cultures employed in the experiment produced hemolysis (beta) in agar plates containing 8 per cent of defibrinated horse blood. All the human streptococci[8] had been grown on artificial media for 2 or more years. All had been isolated from diseased conditions. The bovine strains were isolated by the writer. Five were from cases of mastitis. Two were found in market milk, but were identical in all respects with mastitis streptococci. The equine streptococci, with one exception, were isolated from the nasal mucosa and pharynx of horses suffering from influenza. Equine Streptococcus H.A. 2 was cultivated from an abscess. The low acid-producing streptococci were isolated from market milk. Recent investigations[9] have shown that these may be carried in small numbers in apparently normal udders. Strains M.J. 1 and M. 53 were isolated in 1917. The others were first cultivated in the summer of 1918.

Each tube was inoculated with 0.1 cc. of an 18 hour broth culture. All were incubated at 38°C. for 10 days, when hydrogen ion determinations[10] and titrations against 0.05 N sodium hydroxide were made. Maximum growth was always obtained in the media containing serum. The plain and fermented bouillon cultures were about as vigorous as those observed in the 2 per cent peptone medium. The low acid-producing streptococci from milk grew poorly in media without serum. Serum frequently changes the character of the growth. Strains which grow only at the bottom of tubes in the plain broth frequently produce a marked turbidity throughout the serum medium.

[8] The writer is indebted to Dr. J. Howard Brown, of this Department, for Cultures 32, S.H., S. 8, and 40. Dr. O. T. Avery, of the Hospital of The Rockefeller Institute, supplied Cultures 1, 20, and 24.

[9] Jones, F. S., *J. Exp. Med.*, 1920, xxxi, 347.

[10] Dr. P. E. Howe, of this Department, prepared the standards and indicators for this experiment.

In Tables I to IV the acid production by the different groups of streptococci is given. The figures under the titration columns represent the actual acidity reached in the tubes.

TABLE I.

*Acid Production by Human Streptococci in Plain and Fermented Broths.**

Strain No.	Fermented bouillon.		Fermented bouillon and serum.		Fermented bouillon containing 2 per cent peptone.		Plain bouillon.		Plain bouillon and serum.	
	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.
		per cent		*per cent*		*per cent*		*per cent*		*per cent*
32	5.5	3.6	5.2	4.2	5.7	4.5	5.1	4.2	5.0	4.8
S.H.	5.5	4.2	5.2	4.5	5.5	5.0	5.2	4.0	4.9	4.6
S. 8	5.8	3.9	5.2	4.1	6.0	3.9	5.6	3.2	4.9	4.2
40	5.2	4.2	5.0	4.6	5.4	4.9	5.1	4.1	5.0	4.7
1	5.8	3.5	5.2	4.2	5.8	3.9	5.5	3.3	5.0	4.4
20	5.7	3.1	5.2	4.0	5.7	3.9	5.2	3.2	5.1	4.4
24	5.7	3.1	5.1	4.2	5.7	4.3	5.1	3.7	4.9	4.5

* All the media contained 1 per cent dextrose.

TABLE II.

Acid Production by Bovine Streptococci in Plain and Fermented Broths.

Strain No.	Fermented bouillon.		Fermented bouillon and serum.		Fermented bouillon containing 2 per cent peptone.		Plain bouillon.		Plain bouillon and serum.	
	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.
		per cent		*per cent*		*per cent*		*per cent*		*per cent*
B.M. 1	4.6	6.1	4.6	6.2	4.6	7.0	4.6	5.7	4.6	5.9
C. 59	4.8	5.7	4.8	6.2	5.0	7.0	4.6	6.2	4.6	6.1
B.M. 24	4.9	5.4	4.8	6.1	4.9	6.7	4.6	5.8	4.5	5.7
M. 26	4.8	5.5	4.7	6.3	4.8	7.1	4.6	5.8	4.5	5.7
" 43	4.7	6.0	4.6	6.5	4.7	7.2	4.6	5.9	4.6	5.9
C. 53	4.8	5.3	4.7	6.0	4.8	6.8	4.6	5.5	4.5	5.9
" 67C	4.8	6.2	4.8	6.5	4.8	7.4	4.6	6.0	4.6	5.9

Curves (Text-figs. 1 and 2) constructed from averages of Tables I to IV reveal considerable differences in acid formation in the various media. This is particularly true in the instance of the human and low acid-producing streptococci from milk. It will be observed that

these organisms grown in media low in nutritive material (fermented and plain broth), even though the media contain sufficient carbohydrate, fail to produce as much acid as when a richer medium is employed. The addition of serum, then, not only increases the titratable acid but the ionized acid as well. Thus in the richest medium

TABLE III.

Acid Production by Equine Streptococci in Plain and Fermented Broths.

Strain No.	Fermented bouillon.		Fermented bouillon and serum.		Fermented bouillon containing 2 per cent peptone.		Plain bouillon.		Plain bouillon and serum.	
	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.
		per cent		*per cent*		*per cent*		*per cent*		*per cent*
In. 22	5.1	4.4	5.5	4.4	5.0	5.5	4.9	4.4	5.1	4.6
H.A. 2	5.1	4.3	5.5	3.2	5.1	5.3	4.7	4.4	5.0	4.6
In. 49	5.1	4.5	5.3	5.0	5.1	5.7	4.9	4.4	5.1	4.8
" 14	5.1	3.9	5.4	4.4	5.0	4.8	4.9	3.9	5.1	4.6
" 2	5.1	4.7	5.4	4.2	5.1	5.6	4.9	4.6	5.1	4.5

TABLE IV.

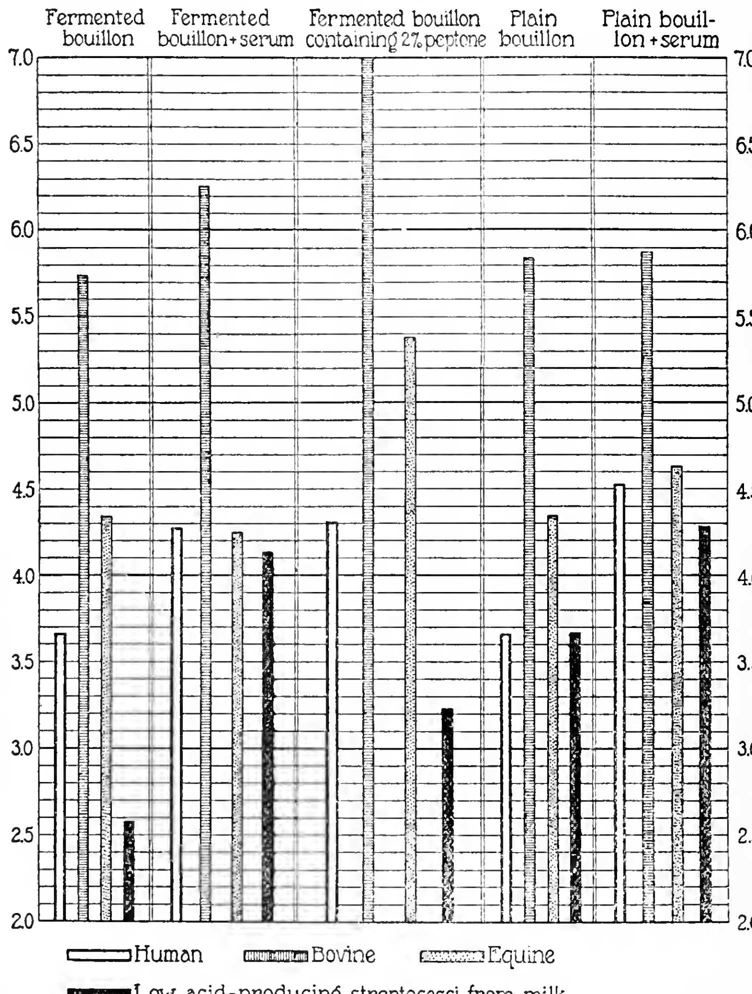
Low Acid-Producing Streptococci from Milk.

Strain No.	Fermented bouillon.		Fermented bouillon and serum.		Fermented bouillon containing 2 per cent peptone.		Plain bouillon.		Plain bouillon and serum.	
	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.
		per cent		*per cent*		*per cent*		*per cent*		*per cent*
M.J. 1	6.3	2.6	5.4	4.1	6.5	3.1	5.2	4.0	5.2	4.0
M. 53	6.3	2.7	5.8	3.5	6.3	2.4	5.8	3.0	5.2	4.1
B.M. 30	6.6	2.0	5.2	4.1	5.9	3.7	5.1	4.0	5.1	4.3
" 22	5.9	3.1	5.1	4.6	6.3	3.7	5.5	3.4	5.1	4.6
" 60	6.3	2.4	5.1	4.4	6.3	3.2	5.5	3.9	5.1	4.4

(dextrose bouillon and serum) the maximum acid production is reached. The sharpest differences are brought out by the low acid-producing milk streptococci; here the greatest increases in acid formation are seen in the serum media. The fermented broth containing 2 per cent peptone apparently offers for the human and low acid-producing streptococci no more favorable media for acid production than that

containing 1 per cent peptone. One would expect the titratable acid to increase on account of the great buffer activity of peptone.

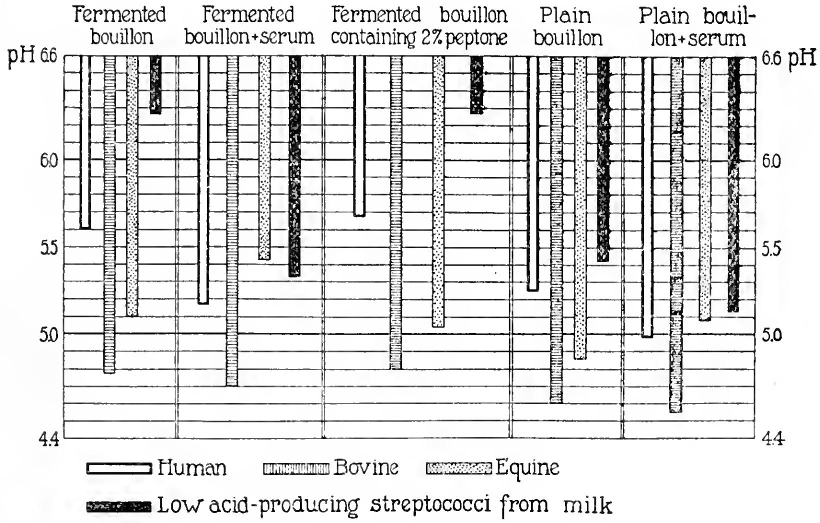
The bovine streptococci follow a somewhat similar curve. Apparently organisms of this type are able to utilize carbohydrate to



TEXT-FIG. 1. Titratable acidity produced by human, bovine, and equine streptococci and low acid-producing streptococci from milk when grown in various media.

a greater degree in media of lower nutritive value. The titratable acidity varies over a considerable latitude; the buffer effect of the 2 per cent peptone is marked. On the whole, there is more acid produced in the serum media.

The reverse is true of the equine streptococci. The organisms apparently produce more acid in media which do not contain serum. Broth containing 2 per cent peptone affords a medium equally as good for acid production as either of the other bouillons to which serum was not added. Two possible explanations for the failure of the



TEXT-FIG. 2. Limiting or final hydrogen ion concentration of human, bovine, and equine streptococci and low acid-producing streptococci from milk when grown in various media.

serum to increase the production of acid suggest themselves. The equine streptococci may not require serum; peptone and meat extracts may be sufficient for all purposes. On the other hand, normal horse serum may contain some substance antagonistic to growth. It is admitted that all strains grew well in the serum media.

The experiment was repeated with the same cultures, but with broths made from the flesh of different calves. When bouillon prepared from the flesh of a calf 6 weeks old was employed much the same results were obtained.

DISCUSSION.

The results obtained readily show that the limiting hydrogen ion concentration may be influenced by differences in the media. The question naturally arises as to the final or limiting hydrogen ion concentration reached by an organism. From present knowledge it may be defined as the acid production in a given medium which finally limits the growth of the organism. That such figures vary over a considerable latitude must be admitted, since human streptococci in one medium may produce acid to the value of pH 5.6 and in another to pH 4.9. Avery and Cullen's figures represent the maximum acid production for human (pH 5.0 to 5.2) and bovine (pH 4.3 to 4.5) streptococci in a medium most favorable for growth. Ayers, Johnson, and Davis' figures (5.5 to 6.0), on the other hand, are given as the final hydrogen ion concentration in a medium of low nutritive value for pathogenic streptococci. While these data were being gathered together the paper of H. Jones[11] appeared. He obtained even greater variations than those recorded in the preceding protocols. He showed that a culture of *Streptococcus hæmolyticus* when grown in glucose broth produced acid to the value of pH 5.11, but when ascitic fluid was added to the bouillon an acidity of pH 4.63 was reached. The increase in acidity failed to vary with an increase in the amount of enriching material. The same limiting hydrogen ion concentration was reached in media to which varying amounts (3 drops to 3 cc.) of ascitic fluid had been added. H. Jones also observed that the initial reaction of the media may influence the limiting hydrogen ion concentration. Thus in concluding it is pointed out that the limiting hydrogen ion concentration of an organism should be defined in terms of media composition, the initial reaction, and other conditions which may favor or hinder abundant growth.

Clark and Lubs[12] noted that methyl red may be destroyed in a short time by active cultures of *Bacillus coli*. Others had observed the same for nitrifying bacteria. Certain streptococci, especially those from sour milk, exhibit this activity to a marked degree while in the active growth phase. The indicator begins to fade within 5 or 10 minutes and at the end of an hour is completely decolorized.

[11] Jones, Horry, *J. Infect. Dis.*, 1920, xxvi, 160.

[12] Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 191.

The advocates of the exclusive application of the hydrogen ion concentration method to bacteriological study have frequently pointed out the unreliability of titration. In studying the tables and charts it will be observed that the differences in titratable acidity are clear-cut. Even the addition of 2 per cent peptone with its buffer effect still reveals great differences in the amount of acid formed by the different groups of organisms. From these experiments one is inclined to believe that titration is equally as satisfactory as the newer method for the study of the fermentative activity of streptococci.

The greatest differences in acid production are brought out in a relatively unfavorable medium, such as dextrose fermented broth containing 1 per cent peptone.

SUMMARY.

The results of variations in acid production in 1 per cent dextrose fermented and unfermented veal broth modified by the addition of 4 per cent of horse serum or 2 per cent of peptone have been recorded. Human streptococci and a group of low acid-producing streptococci from milk produce less acid in the simpler broths (fermented and unfermented). 2 per cent peptone fails to increase the amount of acid produced by these two groups.

The bovine streptococci act much the same as those of human origin. The equine streptococci apparently do not require serum in addition to carbohydrate since they tend to produce less acid in serum media.

The following figures indicate the average minimum and maximum acid production of the various streptococci under the conditions set forth in the experiment.

Human: pH 4.97 to 5.66; titratable acidity 4.51 to 3.66.

Bovine: pH 4.56 to 4.77; titratable acidity 7.0 to 5.74.

Equine: pH 4.86 to 5.42; titratable acidity 5.38 to 4.24.

Low acid-producing streptococci from milk: pH 6.28 to 5.14; titratable acidity 2.56 to 4.28.

ENHANCEMENT OF THE OPSONIZING AND AGGLUTINATING POWERS OF ANTIPNEUMOCOCCUS SERUM BY SPECIFIC PRECIPITATING SERUM.

By IDA W. PRITCHETT.

(*From the Department of Immunology of the School of Hygiene and Public Health, the Johns Hopkins University, Baltimore.*)

(Received for publication, May 3, 1920.)

Bordet,[1] Ehrlich and Morganroth,[2] Pfeiffer and Friedberger,[3] and others have shown that it is possible to produce in animals, through the injection of certain immune or normal sera, antisera capable of inhibiting the action of antibodies. The action of such sera has been ascribed to specific substances designated as anti-antibodies, which are formed when animals of one species are injected with the serum of another species. The serum of the injected animals inhibits the action of antibodies derived from the species whose serum was used as antigen, but is ineffective against antibodies derived from another species. A number of anti-antibodies have been thus produced; *viz.*, antihemolysins, antiprecipitins, antihemagglutinins, anticomplement, and antibacteriolysins. Attempts to produce anti-antitoxins and antibodies for bacterial agglutinins have not been successful. Ehrlich and Morganroth[4] and Wassermann[5] maintain that anti-antibodies for bacterial antibodies cannot be formed in the animal body since animal tissues do not possess receptors suitable for bacterial antibodies. Pfeiffer and Friedberger,[3] however, report experiments in which they succeeded in obtaining a very effective inhibiting serum for cholera vibriolysins.

On the assumption that bacterial anti-antibodies may theoretically be formed in the animal body, the question becomes of interest in connection with the use of therapeutic sera in man, particularly when the same individual is given repeated injections of serum from the same animal species. This condition frequently arises, since therapeutic sera are almost exclusively derived from the horse.

[1] Bordet, J., *Ann. Inst. Pasteur*, 1904, xviii, 591.

[2] Ehrlich, P., and Morganroth, J., *Berl. klin. Woch.*, 1901, xxxviii, 598.

[3] Pfeiffer, R., and Friedberger, E., *Berl. klin. Woch.*, 1902, xxxix, 4; *Centr. Bakteriol., 1te Abt., Orig.*, 1903, xxxiv, 70.

[4] Ehrlich, P., and Morganroth, J., *Berl. klin. Woch.*, 1901, xxxviii, 569.

[5] Wassermann, A., *Z. Hyg. u. Infectionskrankh.*, 1903, xlii, 267.

The observations here reported are the results obtained in experiments on the production of pneumococcus antiopsonins. Second infections due to the same type of pneumococcus, though rare, have occurred. If antiopsonins should arise following serum treatment of the first infection, and persist in the patient's blood, they would tend to neutralize the opsonins in the immune serum employed during the second attack, thus lessening its value. The same effect might theoretically be observed if the patient had at any previous time received normal or non-specific immune horse serum.[1]

As will be seen from the following experiments, no evidence of the formation of pneumococcus antiopsonins could be obtained. On the contrary, the opsonizing and agglutinating properties of the immune horse sera were greatly enhanced in the presence of the sera which were expected to contain inhibiting substances.

EXPERIMENTAL.

Monovalent pneumococcus horse sera, Types I, II, and III, obtained from the Hospital of The Rockefeller Institute, were injected into rabbits. Each rabbit received intravenously 8 cc. of serum of one of the three types, and after 14 days, when the injected serum had disappeared, as indicated by opsonic tests, the animals were bled and their sera collected. Dilutions in salt solution were made of the antipneumococcus horse sera, each equal to one-fourth the dilution finally desired, since equal volumes of whole rabbit serum, pneumococcus culture, and leucocytic suspension were to be added to it. In each of a series of serological tubes was placed 0.1 cc. of the undiluted serum of the injected rabbits, 0.1 cc. of antipneumococcus horse serum dilution, and 0.1 cc. of a 24 hour blood broth culture of the homologous pneumococci. After their contents had been thoroughly mixed by shaking, the tubes were placed in a water bath at 37°C. for 1 hour to allow for sensitization of the pneumococci and interaction of the rabbit and horse sera. At the end of this time there was added to each tube 0.1 cc. of a fresh suspension of leucocytes, obtained by injecting a guinea pig on the previous day intraperitoneally with aleuronat. The contents of the tubes were again thoroughly mixed and incubated for another hour in the water bath, when a film

was made from the sediment in each tube and the slides were fixed in methyl alcohol and stained with Manson's stain. Control experiments were run at the same time in which normal rabbit serum was substituted for that of the rabbits previously injected with pneumococcus horse serum.

To determine whether the order of combination would affect the result, three other methods of combining the various constituents were also employed as follows: (1) Pneumococcus horse serum and precipitating rabbit serum were incubated together for 1 hour before the addition of the pneumococci. After adding the pneumococci the mixture was incubated for another hour before adding the leucocytes. This method permitted considerable precipitation in the rabbit-horse serum mixture before sensitization of the bacteria was begun. (2) Pneumococcus horse serum and pneumococci were incubated for 1 hour before adding the precipitating rabbit serum. After adding the rabbit serum the mixture was incubated for 1 hour before adding the leucocytes. This method permitted sensitization of the bacteria before precipitation was begun. (3) Pneumococcus horse serum and homologous pneumococci were incubated together for 1 hour, when both the precipitating rabbit serum and the guinea pig leucocytes were added and the mixture was incubated for another hour.

No great difference in results was observed with these four methods, so that eventually only the first method, as described in detail above, was employed.

Experiment 1. Type I Pneumococcus Horse Serum.—Type I serum and pneumococci were combined, as described above, with the serum of rabbits previously injected with the Type I serum. The results obtained are presented in Table I.

From Table I it will be seen that the combinations described showed opsonization and microscopic agglutination in dilutions ten times as great as were effective when normal rabbit serum was substituted for that of the injected rabbits, while mixtures of the precipitating rabbit serum and normal horse serum gave uniformly negative results. When the pneumococcus horse serum was replaced by an equal volume of salt solution, neither normal nor precipitating rabbit serum was capable of causing the slightest opsonization or agglutination,

nor did these occur in control mixtures in which both rabbit and horse sera were replaced by salt solution. The Type I horse serum used in this experiment, when diluted with salt solution, showed microscopic agglutination and opsonization of Type I pneumococci in dilutions a little above 1:100.

TABLE I.

*Increased Opsonization and Agglutination of Type I Pneumococci When Combined with the Homologous Immune Serum in a Specific Precipitating Mixture.**

Tube No.	Type I antipneumococcus horse serum and normal horse serum dilutions.	Rabbit 1 (2 wks. after intravenous injection of 8 cc. of Type I antipneumococcus horse serum).				Normal rabbit serum.	
		With Type I antipneumococcus horse serum.		With normal horse serum.		With Type I antipneumococcus horse serum.	
		Aggluti-nins.	Opsonins.	Aggluti-nins.	Opsonins.	Aggluti-nins.	Opsonins.
1	1:100	++++	++++	–	–	++++	++++
2	1:300	++++	++++	–	–	++++	++++
3	1:500	++++	++++	–	–	–	–
4	1:1,000	++++	++++	–	–	–	–
5	1:2,000	++++	++++	–	–	–	–
6	1:3,000	++	++	–	–	–	–

* In this and succeeding tables the degree of opsonization and agglutination is represented by the number of plus signs, ++++ being complete, + slight but definite.

Experiment 2. Type II Pneumococcus Horse Serum.—The technique of Experiment 1 was employed, Type II serum and pneumococci being used. The results are given in Table II.

It will be seen from Table II that the results were essentially the same as those obtained in the experiments with Type I serum and pneumococci. In this particular experiment opsonization was marked in dilutions in which no microscopic agglutination was observed. This, however, was not always found, and was not peculiar to Type II, being sometimes encountered also in experiments with organisms and serum of Type I.

Type III Pneumococcus Horse Serum.—The opsonization and agglutination of Type III pneumococci by the homologous immune

horse serum was not augmented by the addition of the serum of rabbits injected with Type III horse serum, these organisms remaining unopsonized and unagglutinated except in very low dilutions of Type III serum, usually below 1:50.

TABLE II.

Increased Opsonization and Agglutination of Type II Pneumococci When Combined with the Homologous Immune Serum in a Specific Precipitating Mixture.

Tube No.	Type II antipneumococcus horse serum and normal horse serum dilutions.	Rabbit 2 (2 wks. after intravenous injection of 8 cc. of Type II antipneumococcus horse serum).				Normal rabbit serum.	
		With Type II antipneumococcus horse serum.		With normal horse serum.		With Type II antipneumococcus horse serum.	
		Agglutinins.	Opsonins.	Agglutinins.	Opsonins.	Agglutinins.	Opsonins.
1	1:100	++++	++++	–	–	++++	++++
2	1:300	+++	++++	–	–	+	+
3	1:500	++	+++	–	–	–	–
4	1:1,000	+	+++	–	–	–	–
5	1:2,000	–	++	–	–	–	–
6	1:3,000	–	+	–	–	–	–

Experiment 3. Normal Horse Serum.—All the experiments described above have in common the mixture of an immune pneumococcus horse serum with its homologous pneumococci in the presence of a specific precipitating serum. To determine whether the increase in opsonization and agglutination observed in these cases was due to some special property conferred upon the rabbit serum by the injection of the immune pneumococcus serum, or was merely dependent on the presence of the specific precipitating mixture, control experiments were run with the serum of rabbits injected with normal horse serum instead of the immune horse serum. One of these anti-horse rabbit sera had been prepared some months before, but still showed gross precipitins for horse serum in dilutions above 1 : 1,000. Two other anti-horse rabbit sera were prepared with two different samples of normal horse serum, and all three rabbit sera were tested with Type I and Type II pneumococcus horse serum and the homologous pneumococci. The results of these tests were similar for both Type I and Type II. A summary of the findings in an experiment with serum and organisms of Type I is given in Table III which will also serve to illustrate the results obtained with Type II serum and pneumococci.

It will be seen, from a comparison of Tables I and III, that the serum of Rabbit 3, injected some months previously with normal horse serum (Sample A) was but little better than normal rabbit serum in promoting the opsonization and agglutination of Type I pneumococci by the homologous serum, even though a specific precipitating mixture was here present. The other two rabbits, Nos. 4 and 5, injected with Samples B and C of normal horse serum, showed heightened opsonization and agglutination entirely comparable to that observed when Type I horse serum was combined with the serum of rabbits previously injected with the same immune horse serum. Moreover, it was found that the sera of rabbits injected with Immune Horse Serum Type I, II, or III were equally effective in promoting the opsonization and agglutination of Type I and Type II pneumococci by the homologous horse sera, while organisms of Type III remained unaffected by any of the horse-rabbit serum combinations.

TABLE III.

Opsonization and Agglutination of Type I Pneumococci by the Homologous Immune Serum When Combined with the Sera of Rabbits Injected with Normal Horse Serum.

Tube No.	Type I pneumococcus horse serum dilutions.	Rabbit 3 (several mos. after intravenous injection of normal horse serum, Sample A).		Rabbit 4 (2 wks. after intravenous injection of normal horse serum, Sample B).		Rabbit 5 (2 wks. after intravenous injection of normal horse serum, Sample C).	
		Agglutinins.	Opsonins.	Agglutinins.	Opsonins.	Agglutinins.	Opsonins.
1	1:100	++	++	+++	++++	++	++++
2	1:500	+	+	++	++++	+	++++
3	1:1,000	–	–	+	++++	+	++++
4	1:2,000	–	–	+–	++	+	++

The failure of the serum of Rabbit 3 to produce the usual reaction is not understood. The serum of another rabbit immunized at the same time with the same sample of normal horse serum also failed to promote opsonization and agglutination of Type I and Type II pneumococci by the homologous immune sera.

It appears from the foregoing experiments that no special property is conferred upon the serum of rabbits by the injection of immune

horse serum, since no demonstrable antiopsonins are produced by such injections. On the contrary, the serum of such rabbits, as well as that of rabbits injected with normal horse serum, is capable, *in vitro*, of augmenting many fold the opsonizing and agglutinating power of Type I and Type II horse sera for the homologous pneumococci. These findings are in accord with the observations of Moreschi[6] on typhoid agglutinins. He found that when typhoid bacilli were subjected for 2 hours at 37°C. to the action of specific agglutinating serum from various sources (goat, man), and were then washed and resuspended in salt solution, the addition of a small quantity (0.05 cc.) of the serum of rabbits previously injected with the same agglutinating serum raised the titer of the immune serum five- to tenfold. His explanation for this phenomenon was that the serum of the injected rabbits, while itself incapable of agglutinating typhoid bacilli, combined with and precipitated the agglutinating serum already absorbed by the bacteria, thus causing agglutination. Non-specific bacteria, such as the cholera vibrio and *Bacillus dysenteriæ*, he found to be unaffected by the serum mixtures, while the precipitating serum had no affinity for unsensitized typhoid bacilli. The last two observations were also borne out by the present experiments, since in no case were Type I and Type II pneumococci opsonized or agglutinated by a heterologous or normal horse serum, even in the presence of a specific precipitating serum.

Protection Experiments.

Since the efficacy of therapeutic pneumococcus serum (Type I) depends largely on its opsonizing power, and since this power is so greatly enhanced *in vitro* by the addition of a specific precipitating serum, it was hoped that a corresponding increase in protective power might be produced by the same means. Experiments were accordingly carried out for the purpose of testing this point, mice being chosen as the test animals. The results obtained were disappointing. In this instance opsonization in the test-tube was not paralleled by protection in the animal, since no very marked increase in protection could be demonstrated. The impossibility of maintaining *in vivo*

[6] Moreschi, C., *Centr. Bakteriol., 1te Abt., Orig.*, 1908, xlvi, 456.

the same quantitative relations between bacteria and serum that exist *in vitro* may account for the failure. Dilution of the opsonizing and precipitating sera with peritoneal exudate and unequal absorption of the various constituents would tend to upset the balance very quickly, and no doubt would markedly affect the outcome. Incubation of the mixtures before injection served only to confuse the results, since the pneumococci were sometimes injured by the broth and died, while they multiplied in the tubes containing serum, so that not infrequently the control animals outlived the other mice. Only once was

TABLE IV.

*Protection Afforded to Mice from a Combination of Type I Pneumococcus Horse Serum with Specific Precipitating Rabbit Serum.**

Mouse No.	Weight.	Type I antipneumococcus horse serum.	Normal horse serum.	Normal rabbit serum.	Serum of rabbits previously injected with Type I horse serum.		Broth.	Type I pneumococcus broth culture.	Result.
					No. 1.	No. 6.			
	gm.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	
1	20	0.0005		0.25				0.01	Died in 33 hrs.
2	20	0.0005			0.25			0.01	Survived.
3	20	0.0005				0.25		0.01	"
4	20	0.0005					0.25	0.01	Died in 22 hrs.
5	20		0.0005		0.25			0.01	" " 22 "
6	20		0.0005			0.25		0.01	" " 22 "

* The total volume of each dose was made up to 1 cc. and injected as soon as mixed into the peritoneal cavity, without incubation. The serum samples of Rabbits 1 and 6 were a month old at this time.

a successful experiment performed, in which the mice survived that had received a mixture of pneumococcus horse serum and specific precipitating rabbit serum, while all the other mice died. The protocol is presented in Table IV.

DISCUSSION.

From the foregoing experiments it appears that a simple precipitating mixture is capable of augmenting the opsonization and agglutination of Type I and Type II pneumococci by their homologous immune

sera. Similar observations have been made by other workers with other types of antibodies. Friedberger and Moreschi[7] found that rabbit red cells sensitized with goat amboceptor were much more readily hemolyzed in the presence of a small quantity of the serum of a rabbit previously injected with the goat amboceptor. Arkwright,[8] in his experiments on the agglutination of *Bacillus typhosus*, found that in a specific serum precipitating mixture, with bacterial extract as antigen, not only the specific bacteria, but even non-specific bacteria and inanimate particles were agglutinated. Nicolle[9] also reports that non-specific bacteria are carried down when present in a specific serum precipitating or agglutinating mixture. He found that in mixed suspensions containing equal numbers of *Bacillus coli* and *Bacillus typhosus*, the addition of an agglutinating serum specific for either would completely agglutinate both. The phenomena of non-specific opsonization and agglutination have not been observed in the present study, since neither Type I nor Type II pneumococci were opsonized or agglutinated in the presence of the heterologous immune serum or of normal horse serum, even though the addition of the anti-horse rabbit serum furnished a specific precipitating mixture. Arkwright's explanation for his results is that the coagulum formed in the serum precipitating mixture drew together and clumped the bacteria, specific and non-specific, a mechanical explanation that could hardly be applied to opsonization and phagocytosis. Since the reaction of an antigen with its specific antibody results in lowering the surface tension of the mixture,[10] and since it is thought that phagocytosis may be a surface tension phenomenon,[11] the explanation for the increased opsonization of Type I and Type II pneumococci by their homologous immune sera, in the presence of a specific precipitating serum, may perhaps lie in the changes in surface tension thus produced, though why a general lowering of the surface tension of the

[7] Friedberger, E., and Moreschi, C., *Centr. Bakteriol., 1te Abt., Orig.*, 1908, xlv, 346.

[8] Arkwright, J. A., *J. Hyg.*, 1914, xiv, 261.

[9] Nicolle, C., *Ann. Inst. Pasteur*, 1898, xii, 161.

[10] Wells, H. G., Chemical pathology, Philadelphia and London, 3rd edition, 1918, 208.

[11] Wells,[10] pp. 266–275.

medium should render the leucocytes more actively phagocytic is not clear. Another possibility is that precipitation takes place in the serum already absorbed by the sensitized bacteria, or that the previously precipitated substance is adsorbed by the bacteria together with the specific opsonins, and that these adsorbed substances render the pneumococci more susceptible to phagocytosis. It seems probable that the effect, whatever it may be, is exerted upon the bacteria, but at present no satisfactory explanation for the phenomenon has been reached.

SUMMARY.

1. No demonstrable antiopsonins are formed in rabbits following the intravenous injection of monovalent pneumococcus horse sera, Types I, II, and III.

2. The serum of rabbits injected with immune pneumococcus horse serum, Type I, II, or III, or with normal horse serum, when mixed in the proportion of 1:4 with Type I or Type II pneumococcus horse serum, can greatly augment, *in vitro*, the opsonization and agglutination of Type I and Type II pneumococci by the homologous immune horse sera. No similar effect is obtained with Type III serum and pneumococci.

3. The increase in opsonization and agglutination is dependent upon (*a*) specific sensitization of the pneumococci by the homologous immune serum and (*b*) the presence of the precipitating serum. In the absence of sensitization, as when a heterologous or normal horse serum is employed, opsonization and agglutination do not occur, even though a precipitating mixture is provided. The substitution of normal rabbit serum for the precipitating rabbit serum gives opsonization and agglutination in dilutions slightly higher than are effected with salt solution only, due possibly to the more favorable medium created for the leucocytes by the addition of 25 per cent of whole rabbit serum.

4. Different methods of combining the immune horse serum, precipitating rabbit serum, and pneumococci yield very similar results, preliminary sensitization of the bacteria before precipitation, or precipitation in the rabbit-horse serum mixture before the addition

of the pneumococci for sensitization causing little if any difference in result from that obtained when immune horse serum, precipitating rabbit serum, and pneumococci are all mixed and incubated together.

5. This increased opsonization in the test-tube does not seem to be paralleled by increased protective power, or at any rate such protection is not readily demonstrated.

USE OF THE SINGLE CELL METHOD IN OBTAINING PURE CULTURES OF ANAEROBES.

By M. A. Barber, Ph.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plate 23.

(Received for publication, May 7, 1920.)

The object of this work has been to determine to what extent the pipette method may be of service in the isolation of anaerobes. No question is raised as to the possibility of isolating anaerobes by plating or by any other of the methods ordinarily in use, but such methods are often time-consuming and sometimes leave the worker in doubt as to whether or not the culture is absolutely pure. Difficulties in isolation are especially great with mixtures of certain anaerobes, and doubt has been thrown on the results of some of the earlier workers because of the probability that their cultures were impure. The pipette method is relatively simple and rapid, and affords the worker the satisfaction of knowing that his culture is the progeny of a single cell. Further, it offers some advantages in the study of anaerobiosis, variability, motility, rate of growth, and behavior in a medium of small sowings exactly known.

Technique.

The technique followed in this work has been essentially that described for aerobes, except that the isolated organisms have been subsequently grown under anaerobic conditions. The pipette method has been fully described (1), and only such details will be given here as should be especially emphasized or are especially applicable to work with anaerobes.

The size of the isolation chamber may vary according to the needs of the investigator, but I have found most convenient one about 7 cm. long, 3.25 cm. broad, and 2 cm. high, which gives abundant working room and allows the use of a large cover-glass, 60 by 35 mm. The

preparation of the cover-glass is a matter of considerable importance, especially when colonies are to be grown on it. Carefully cleaned covers are smeared with vaseline and kept in stock ready for use. Just before use most of the vaseline is removed by holding the cover slantwise under the hot water tap. It is then washed with alcohol and again held under the tap. Finally, it is covered with alcohol and wiped clean with a cloth. It is then perfectly clear to the eye, but retains the very small film of vaseline so necessary to the preparation of small droplets. Prepared cover-glasses may be sterilized by dry heat or over the flame, care being taken to avoid heat sufficient to burn off the vaseline.

The point of the pipette should be made with an opening large enough to admit organisms readily, but not so large as to make it difficult to isolate the organisms in very small droplets. A diameter about equal to the average length of a spore-containing bacillus of *Bacillus tetani* is very satisfactory. It will be found most convenient to make very fine pointed closed pipettes and to break off portions of the point in a drop of broth under microscopic control until an opening of convenient size is obtained. A small quantity of broth should be allowed to enter the pipette before isolation is begun.

Certain doubts which have been raised as to the possibility of isolating a single organism in a hanging drop must arise from a misapprehension of the pipette method (2). The droplets containing the single organism may be made very small, and a mere film of liquid is present on the cover-glass, not a large rounded drop in the margins of which a second organism may lie concealed. The photomicrograph of a spore-bearing bacillus of *Bacillus tertius* (Fig. 1) illustrates the character of the droplet. This droplet has spread somewhat, owing to moisture condensed during the process of photography, and the small particles seen in it are probably due to the excess of vaseline used in the preparation of a specimen suitable for longer preservation during photography. In routine work the organism may be isolated in perfectly clear fluid and in droplets somewhat smaller than that shown in the illustration. After a series of organisms has been isolated, each droplet may be gone over with the oil immersion lens while the cover is on the isolation chamber, or, if the light there is insufficient, when temporarily removed to a shallow moist chamber. One cannot, of

course, exclude the possibility of the presence of any organism not visible to the oil immersion lens, but this limitation is common to all methods of isolating living organisms. If desired, the organism may be washed and rewashed in sterile broth, a procedure useful in removing any growth substances brought over from the original culture, but no guarantee against the inclusion of an invisible living substance. One may observe the first stages of development of an organism in a small drop of broth or at the margin of an agar droplet. In Fig. 2 a young colony of *Bacillus sporogenes* is shown growing out from the original position of a spore at the margin of an agar droplet.

The isolated organisms may be immediately transferred to test-tubes or grown on the cover-glass until colonies have formed and these then transferred. In transferring the organisms immediately after isolation the isolating pipette is discarded, and by means of new pipettes—a fresh one for each organism—the organisms are separately transferred to test-tubes. Each new pipette is supplied with a small amount of sterile broth before use, preferably from recently boiled broth or semifluid agar. In taking up an organism it is important to make sure that it enters the pipette and does not remain clinging to the margin of the opening. This precaution is not absolutely necessary in the case of aerobes or of anaerobes which are transferred to a liquid which is subsequently freed from air, but when a transfer to deep agar or to broth protected by a layer of vaseline or oil is intended, an organism not well within the pipette may remain on the vaseline or at the surface of the agar. In order to provide sufficient liquid for washing the organism well back a very small drop of broth may be made with the pipette in the immediate vicinity of the isolated organism and a small amount of broth added to the drop containing the isolated spore or bacillus just before it is taken up. The organism is then usually seen to enter the mouth of the pipette, and the additional droplet of broth is taken up in order to wash it well back. Sometimes a little manipulation is required to free an organism from substances which tend to make it cling to the film of surface tension of the droplet. Most bacilli and spores still enclosed in the mother cell give little trouble in this respect. When the tip of the pipette is brought to the desired depth in the medium in the test-tube, enough broth should be discharged to insure the

exit of the organism, and the discharge should be stopped before air is introduced.

With bacilli of some species it is desirable to minimize their exposure to the air during isolation. In this case it is well to introduce the isolating pipette directly into the culture from which isolations are to be made. The pipette is adjusted in the holder, a few isolations are made, and the organisms are transferred to an anaerobic environment as quickly as new pipettes can be prepared. Exposure to the air can be limited to 4 or 5 minutes, and the time of exposure can be fairly accurately measured. When pipettes for transferring the isolated organism are made in advance, or when two pipettes are used simultaneously, one for isolating and the other for taking up the organism, the time of exposure to air may be still further limited. No diluting fluid is needed in making isolations if bacilli are taken from a young culture just becoming cloudy.

Seeding Material.

In this work the aim has been to purify cultures isolated by the usual means. It might be possible to use the pipette method in isolating directly from the original source in cases in which the spores of anaerobes predominate and a considerable proportion of them are viable; but, in any case, it would be more convenient to make a preliminary isolation by one of the ordinary means. Spores for seeding were usually taken at maturity or within a few days afterwards, but in some instances spores from cultures 2 to 4 months old were sown and a fair proportion of positive results was obtained. In order to obtain proper bacilli for isolation, media were fairly heavily seeded, and the isolations made at the time when the first cloudiness appeared, usually within 4 or 5 hours. At this period nearly all the bacilli are living, and the longer elements or filaments which are then usually present are more easily isolated and possibly give a better chance of growth. In a few cases positive results were obtained with bacilli from cultures 24 hours old or more. In this paper the term "single bacillus" is made to include, in some cases, short filaments which may have been composed of several united bacilli.

Anaerobiosis.

In part of our series (Tables I and II, columns marked*) organisms were transferred to a fluid medium and the air was subsequently removed by means of an air pump. In the majority of cases boiling alone without vacuum was relied on to remove oxygen, and with

TABLE I.

Growth of Single Bacilli in Test-Tubes.

Species.	Medium and proportion of positives.													Total, all media.
	Glucose broth.	Serum glucose broth.	Semisolid glucose agar.	Semisolid serum glucose agar.	Semisolid serum agar.	Firm glucose agar.	Firm Veillon agar.	Liver peptone agar.	Minced brain.	Liver peptone water under vaseline.	Liver peptone water in vacuum.*	Milk.*	Plain broth.*	
B. sporogenes	2/14					0/6								2/20
" *welchii*	10/25		0/3						0/1	0/7	3/6	1/3	0/1	14/46
" *tetani*	13/36		3/12				1/3				0/5			17/56
" *œdematis*	3/12													3/12
" *botulinus*	3/6						1/3				0/5			4/14
B. of Ghon Sachs	0/16		1/7			0/1		0/6		1/6				2/36
B. aerofœtidus	1/3	0/1	1/2								0/23			2/29
" *putrificus*	0/2	1/2		1/3										2/7
" *bellonensis*	0/3	0/3	1/2	0/3										1/11
" *tertius*	0/1	2/3	0/2	0/2	3/3									5/11
" *fallax*	0/8			0/2	1/2									1/12
" *œdematiens*	2/3	0/2	1/1											3/6
" *bifermentans*	1/12	0/1	0/1	0/1	0/4					0/1				1/20
" *histolyticus*	1/1			1/3						2/2				4/6
Vibrion septique	0/47	0/8	1/37	0/12	0/7					0/3				1/114
Total, all species.	36/189	3/20	8/67	2/26	4/16	0/7	2/6	0/6	0/1	3/19	3/39	1/3	0/1	62/400

* Air removed after inoculation by means of an air pump.

fluid media access of air was prevented by a layer of vaseline 1.5 or 2 cm. thick. Immediately before inoculation the upper part of the vaseline layer was liquefied by gentle heat. Both firm and semisolid agar was used without any layer of vaseline or other protecting substance, and the isolated organism was introduced deep into the medium (Fig. 3). Except with serum-containing media, tubes were, as a routine, boiled and quickly cooled before use.

A simple method of anaerobiosis was found adequate for growing spores in hanging drops. A shallow moist chamber about 45 mm. long, 25 mm. broad, and 2 mm. deep, inside measurement, is made by cementing strips of glass to a large slide. Organisms are isolated in an area of about 3 by 1 cm. in the center of a large cover-glass. The isolated spores are arranged about 1.5 or 2 mm. apart, and enough medium is added after isolation to provide each spore with a droplet

TABLE II.

Growth of Single Spores in Test-Tubes.

Species.	Medium and proportion of positives.													Total, all media.
	Glucose broth.	Serum glucose broth.	Semisolid glucose agar.	Semisolid serum glucose agar.	Semisolid serum agar.	Firm glucose agar.	Firm Veillon agar.	Liver peptone water under vaseline.	Liver peptone water in vacuum.*	Milk.*	Plain broth.*	Egg cube.*	Meat.*	
B. sporogenes.......	11/16	2/5		3/6		2/5		5/6		1/1	1/2	1/1		26/42
" *tetani*...........	7/17		3/6	2/6			0/5		0/14					12/48
" *botulinus*........							0/3		1/12					1/15
B. of Ghon Sachs...	3/3		1/3											4/6
B. putrificus.......				4/5						1/1	1/1	1/1	1/1	8/9
" *bellonensis*......		0/2	0/5	4/5	0/3				2/6					6/21
" *tertius*..........				10/12	1/1				1/7					12/20
" *œdematiens*......				4/5					3/7					7/12
" *bifermentans*.....			3/3	4/6					1/3					8/12
" *histolyticus*......									2/11		0/2			2/13
Vibrion septique....			1/1	3/3			0/4		3/5					7/13
Total, all species.	21/36	2/7	8/18	34/48	1/4	2/5	0/12	5/6	13/65	2/2	2/5	2/2	1/1	93/211

* Air removed after inoculation by means of an air pump.

about 1 mm. in diameter, although this quantity may be made to vary, as desired. Larger drops sown with many organisms are also made for comparison. After the organisms have been isolated and supplied with medium, the under surface of the cover-glass, with the exception of the area in the center occupied by the isolations, is covered with soft glucose agar taken from the surface of a culture of *Bacillus pyocyaneus* about 4 or 5 hours old. This culture should contain many actively growing organisms, and a little fresh medium

may be added to it just before spreading. The area in the center of the cover may be enclosed in a ring of soft paraffin in order to prevent any danger of the spread of the *pyocyaneus* to the isolated anaerobes. The bottom of the moist chamber is supplied with a similar layer of *pyocyaneus* culture. The entire surface of the bottom may be covered, but better light is afforded for observation and there is less danger of condensation of an excess of moisture on the cover-glass if an area at the center of the bottom is left free. The cover is immediately sealed on the moist chamber by means of vaseline, and the chamber is enclosed in a Petri dish before it is placed in the incubator.

Pyrogallic acid and potassium hydroxide, so arranged that they may be mixed in the bottom of the moist chamber after the cover has been sealed on, were used alone and in connection with the *pyocyaneus* culture, but appeared to offer no advantage.

Preparations growing on the cover may be examined at any stage of growth and returned to the incubator without disturbing the cover-glass. Moist chambers taken out of the incubator should not be exposed to room temperature while still warm, since the cover-glass cools more rapidly than the slide and may accumulate an oversupply of moisture of condensation. It is best, then, to allow the preparations to attain room temperature before removing them from the Petri dish.

Media.

The media employed are shown in Tables I and II. Glucose media contained for the most part 0.5 per cent of glucose and were adjusted to a pH of approximately 7.4. Serum media contained 3 to 5 per cent of unheated rabbit serum, or, in a few instances, horse serum. The liver peptone water consisted of Dunham's peptone without salt adjusted to a pH of 7.8. The media were tubed, and to each tube a small piece of liver was added. It was then autoclaved, and after autoclaving the reaction was found to be about pH 7.4. Semi-solid agar media were made soft enough to be easily drawn into a fine pointed pipette while cold. As a routine, test-tubes of narrow diameter were used, and 10 cc. of media supplied to each tube.

Proportion of Positive Results Obtained from One-Cell Sowings in Test-Tubes.

Table I shows the number sown and proportion of positives of single bacilli sown into various media in test-tubes, and Table II gives similar data for spores. The denominators of the fractions in these and all subsequent tables give the number sown; the numerators, the number positive. Fifteen species, or possible species, are included. Questions as to the possible identity of certain species, as the bacillus of Ghon Sachs and *vibrion septique*, need not be discussed here. There were four strains of *Bacillus tetani*, one of them a Type III, two strains of *Bacillus botulinus*, and two of *Bacillus welchii*, one of which had been recently isolated from feces. For the most part the various strains had long been grown on nutrient media. In all strains of all species positive results were obtained either from single spores or from single bacilli, and in a majority of species from both. In the aggregate, 211 sowings of spores gave 44.1 per cent positive, while 400 sowings of bacilli gave only 15.5 per cent positive. The poorer showing of the sowings of bacilli is mainly due to the large number of negatives occurring in three species, *Bacillus aerofœtidus*, the bacillus of Ghon Sachs, and *vibrion septique*. The last two named gave positives with single spore sowings on the first trial.

As a routine, isolations were done in batches of five or six transfers. In a majority of species the first trial gave at least one positive among the first four tubes inoculated. In nearly 70 per cent of the thirty-six spore groups positives were obtained in at least one of the first four tubes inoculated. Fourteen spore groups, including nine species and ten strains, were sown into semisolid glucose agar, part with and part without serum. Of these, thirteen gave positives in at least one of the first four tubes inoculated. To isolate six spores or bacilli and transfer them to test-tubes requires, as a rule, less than 1 hour. These data give a better idea of the practicability of the method and the probabilities of success than do the percentages given in the tables, which include all experiments, tentative and otherwise.

Hardly enough experiments were made with all the different media to offer a fair basis for estimating their comparative value. The various glucose media served about equally well for bacilli, and semi-

solid serum glucose agar gave the best results for spores. Semisolid agar media not only give a relatively high proportion of positives, but offer the most convenient form for one-cell inoculations, since they do not need to be liquefied for inoculation and require no protective layer of vaseline or oil. Further, if positives are examined early one may often observe the formation of a single colony at the point where the organism was discharged. Certain advantages of semisolid media for routine work with anaerobes have been described by Lignières (3).

Bacteria in positive cultures in all media were examined microscopically, gas formation and other characteristics noted, and transfers made to broth or other media under aerobic conditions, in order to detect possible contaminations with air organisms. Such contaminations during the manipulations occur so rarely and are so easily detected that they constitute no source of difficulty in the pipette method.

Growth of Isolated Organisms in Hanging Drops.

The method of anaerobiosis for preparations in hanging drops has been described. Single spores from eight species were successfully grown. The media used were chiefly semisolid glucose agar, with and without serum, and serum glucose broth. The species tested and the proportion positive were as follows: *Bacillus tetani*, 17/57; bacillus of Ghon Sachs, 1/7; *Bacillus sporogenes*, 42/50; *Bacillus putrificus*, 1/8; *Bacillus bellonensis*, 11/24; *Bacillus œdematiens*, 4/12; *Bacillus tertius*, 10/19; *Bacillus bifermentans*, 1/29. Total, 87/206, or 42.2 per cent.

In some batches of all species single spores from the same source were sown at the same time into about 10 cc. of a similar medium in test-tubes. The test-tubes gave a total of 55.4 per cent positives out of 65 sown, while the corresponding hanging drops gave 38.2 per cent positives out of 102 sown.

The hanging drop method has certain advantages; isolations are quickly made—$\frac{3}{4}$ hour or less suffices for a series of ten isolations—and only two pipettes are required for a whole series, one for isolation and one for supplying additional media to the droplets. Early growth, variability, motility, and spore formation may be conveniently observed.

With species which readily form spores, it would probably save time in isolation to sow spores in hanging drops in preference to the direct transfer to test-tubes. The strains which fail to grow on the cover-glass could be reserved for the other method. Lag occurs in the hanging drop as in test-tubes, and preparations should not be discarded if no growth occurs on the 1st day. When colonies have formed in the hanging drop they may be easily transferred to the test-tube. In most species spores formed readily in the hanging drop, and these spores have afforded convenient material for further isolations.

The hanging drop method has not proved successful for growing isolated bacilli, although a large number of bacilli sown in one drop will often grow. A number of attempts to grow young isolated bacilli of several different species gave uniformly negative results, possibly because of too long exposure to air before sufficient anaerobiosis was attained.

Growth of Spores of Bacillus tetani in Homologous Serum.

The growth in hanging drops of single tetanus spores in a medium containing tetanus antitoxin was compared with similar sowings in a control medium containing no antitoxin. Both media consisted of semisolid glucose agar containing 3 per cent horse serum. The antitoxin serum contained approximately 325 units per cc., and the tetanus spores were taken from the strain used in immunization. Growth occurred in three out of ten sowings in the antitoxin medium, and in two out of ten controls. In a series sown in serum glucose broth the antitoxin medium gave one out of four positive, and the control one out of five. Spore formation and the quantity and character of the growth were similar in the two media, except that in the antitoxin medium bacilli showed a greater tendency to adhere in chains. Sowings of single spores in quantities of 10 cc. of the antitoxin medium gave two positive out of three sown. One of these positives is shown in Fig. 3.

Effect of Exposure to Air on Isolated Bacilli and Spores.

In the course of routine work the period of exposure to air was noted in many groups of experiments, and the proportions of positives occurring after various periods of exposure are shown in Table III. Only batches in which at least one positive occurred and only those in which young bacilli were sown are included in this table. The bacteria were exposed to the air in the very small droplets of the liquid, usually glucose broth, in which they had been grown, and the period of exposure is reckoned from the time when a bacillus issued from the isolating pipette until it was deposited in the test-tube under anaerobic conditions. To the time of full exposure on the cover-glass one should add, theoretically, the time necessary for the reduction of any free oxygen introduced with the organism into the test-tube. However, the time necessary to attain sufficient anaerobiosis for growth must be short, since a very tiny amount of liquid is brought into 10 cc. of medium.

TABLE III.

Effect of Exposure to Air on Young Bacilli.

Species.	Length of time exposed to air and proportion of positives.									Total, all periods.
	4–6 min.	7–10 min.	11–17 min.	18–23 min.	24–30 min.	31–36 min.	37–43 min.	44–60 min.	61–72 min.	
B. welchii	3/3	1/2	3/5	1/3	0/4	0/2	0/2			8/21
" *tetani*	3/7	3/4	3/7	3/10	1/4	1/5	1/3	1/7	0/1	16/48
" *œdematis*	2/2	1/1		0/3	0/1	0/2	0/2	0/1		3/12
" *botulinus*	1/1	0/1	1/1	0/2	1/1					3/6
" *sporogenes*	0/1	1/1	0/1	0/1	1/1	0/1				2/6
B. of Ghon Sachs	2/2	0/1	0/1	0/1	0/2					2/7
B. aerofœtidus	1/3	1/2	0/1							2/6
" *putrificus*	2/3	0/3	0/1							2/7
" *bellonensis*	1/3	0/2								1/5
" *tertius*	2/2	3/3	0/1							5/6
" *fallax*	1/2	0/2	0/2							1/6
" *œdematiens*	1/2	1/2	1/2							3/6
" *bifermentans*	1/3	0/3	0/2							1/8
" *histolyticus*	2/2	2/2	0/2							4/6
Total, all species	22/36	13/29	8/26	4/20	3/13	1/10	1/7	1/8	0/1	53/150
Percentage positive	61.1	44.8	30.8	20.0	23.1	10.0	14.3	12.5	0	35.3

It appears from Table III that, on the whole, the probability of growth of young bacilli decreases with the increase in the time of exposure to the air, and that different species vary somewhat in sensitiveness to oxygen, as far as can be judged from the comparatively small numbers under each.

The results obtained with *vibrion septique* are not included in this table, since a different method of isolation was used for the batch in which a positive was obtained. Nearly 100 trials with sowings of young bacilli of *vibrion septique* were made by the usual method without success. The organisms were living when isolated, since in many cases the bacillus was distinctly motile, although motility ceased after an exposure to the air of $\frac{1}{2}$ or $\frac{3}{4}$ minute. A great variety of media was employed. The addition to glucose broth of salicin, of unheated pieces of rabbit kidney, and of broth from filtered young cultures of *vibrion septique* and of *Bacillus welchii* gave no success. It hardly seems likely that the medium was at fault, since single spores of this species grew readily in semisolid serum glucose agar—in one series of four all were positive. Furthermore, larger sowings of young bacilli grew rapidly in every medium tested. The time of exposure to air was cut down to $1\frac{1}{4}$ to 2 minutes by the employment of transfer pipettes prepared in advance and by the simultaneous use of two pipettes, but no growth was obtained. Sowings of ten or fifteen bacilli after an exposure to the air of 4 to 6 minutes also gave negative results.

Finally, a series of isolations was made under sterile paraffin oil spread on the cover-glass. The first oil series gave one positive out of seven sown. The organism which grew was motile when transferred. A second paraffin oil series of eight transfers gave all negatives, and all of a series of fourteen isolated under vaseline or vaseline diluted with paraffin oil were also negative. Motility persisted somewhat longer under vaseline than in similar droplets exposed to the air. In small hanging drops made in an atmosphere from which oxygen had been removed by pyrogallic acid and potassium hydroxide motility persisted for an hour or more.

It seems probable that young bacilli of *vibrion septique* are especially sensitive to free oxygen, and that this sensitiveness explains in large part the negative results obtained after the transfer of single bacilli.

Our experiments tend to confirm those of Bachmann (4), who found the vegetative forms of certain anaerobes very sensitive to an exposure to air of only 10 minutes plus the time required to obtain full anaerobiosis subsequently. In Bachmann's experiments bacilli were exposed to the air in agar plates, and the plates were subsequently brought under anaerobic conditions in which organisms remaining viable might form colonies.

In our experiments spores showed no reduction in the proportion of positives as the result of exposure to air during periods as great as 85 minutes at least. In 61 one-spore sowings, in which the time of exposure to air was noted, the periods of exposure and the proportion of positives were as follows: 12 to 17 minutes, 2/3; 18 to 23 minutes, 2/6; 24 to 30 minutes, 6/9; 31 to 36 minutes, 6/8; 37 to 43 minutes, 5/9; 44 to 60 minutes, 6/10; 61 to 72 minutes, 4/10; 73 to 85 minutes, 3/6. Total for all periods, 34/61. These results represent the aggregate of six species and include only batches in which at least one positive occurred.

The effect of exposure to air on anaerobes was not made the subject of a special study in our experiments, but only as incidental to the technique of isolation. It would appear that in the isolation of bacilli one should minimize the exposure to air as far as possible, while the viability of spores is not appreciably affected by an exposure of an hour or more.

Lag.

In a great majority of the experiments the inoculated test-tubes were not enclosed in an anaerobic jar, and the time of first appearance of growth could be approximately noted. In all positives occurring in tubes in which bacilli were sown, growth appeared on the day following inoculation. The first appearance of cloudiness in broth or of a colony in agar varied approximately from 16 to 21 hours after sowing. Growth from single spore sowings showed a marked tendency to lag. The amount of lag by days is shown in Table IV. A day was reckoned to the time of the last observation, usually about 5 p.m. If growth appeared on the 1st day, it was usually later than in sowings of single bacilli of the same species. It is seen from Table IV that about 37 per cent of the positives appeared on the 2nd day or later,

thus showing a lag of a day or more. The condition of the spores sown is doubtless an important factor. This fact is illustrated by the results with the washed spores of *Bacillus tertius*. Spores were twice washed by centrifugation in normal salt solution, and two of the three positives lagged 3 days or more. Three grew out of a series of five sown. Unwashed spores from the same source gave all positives, and all grew without lag. In addition to the washing the spores were kept several hours in salt solution at refrigerator temperature.

TABLE IV.

Time of Appearance of Growth from Single Spores Sown in Various Media in Test-Tubes.

Species.	Total No. of positives.	Day following inoculation on which growth appeared and incidence of positives.						
		1	2	3	4	5	6	8
B. sporogenes	20	6	12	2				
" *tetani*	12	9	2		1			
" *putrificus*	6	4	2					
B. of Ghon Sachs	4	4						
B. bellonensis	4				2		1	1
" *œdematiens*	4	4						
" *tertius* (unwashed spores)	8	8						
" " (washed ")	3	1			1	1		
" *bifermentans*	7	7						
Vibrion septique	4	2		1	1			
Total	72	45	16	3	5	1	1	1

The marked tendency to latency observed in the growth of the spores of some species illustrates one source of difficulty in separating these from other species by plate methods.

Variability in Morphology of Bacillus sporogenes.

The method employed in isolation is available for a study of variability in anaerobes as in aerobes, as is shown by the following experiment. Four spores from a culture of one-cell origin were isolated and sown in hanging drops. Of the four colonies formed, one showed a marked tendency to the formation of filaments, these appearing in the form of a fine network. Six spores from the filamentous colony

were isolated in a new series of hanging drops, and all showed the same filamentous tendency to a greater or less degree as compared with a series of controls from a non-filamentous source. In further series on a different medium the filamentous strain reverted to normal. A long filament isolated and transferred directly to a test-tube gave the same type of growth as controls.

DISCUSSION.

It is a matter of common knowledge that large sowings of bacteria into a new medium give a better chance of growth than small sowings. Large sowings offer a larger assortment of individuals, some of which may be more vigorous or otherwise better capable of adapting themselves to new conditions, and, furthermore, any growth products carried over with the bacteria may favorably modify the new environment. When a single cell is sown the change in environment is more complete, and unless the new environment is identical with the old a process of adaptation may be necessary. In the experience of the writer, single aerobes have often given 100 per cent of positives when sown into as much as 10 cc. or more of new medium favorable to the species sown.

The importance of employing a suitable medium is shown by experiments on Pneumococcus Type I. Two batches of plain broth made during successive weeks and apparently similar in constituents and reaction were compared. Six tubes of Batch 1 and six of Batch 2 were alternately sown with pneumococcus taken at the height of growth. Tubes of Batch 1 received each one pair of pneumococcus, and those of Batch 2 received each one to four pairs. All the tubes of Batch 1 showed abundant growth which proved to be pure culture of pneumococcus, while all the tubes of Batch 2 remained sterile. All the tubes contained 10 cc. of medium. A flask containing 75 cc. of Broth 1 also gave an abundant growth with a sowing of one pair. Large sowings in Broth 2 grew readily.

When single anaerobes are sown, the lack of proper conditions of anaerobiosis may offer more obstacles to growth than is the case when larger sowings are employed. Burri and Kürsteiner (5) have shown that the growth of *Bacillus putrificus* initiated under strict anaerobic

conditions may continue with increased activity under conditions of aerobiosis unfavorable to the beginning of growth.

In our experiments 100 per cent of positives were obtained with certain lots of five or six tubes each in which single spores of *Bacillus tertius*, *Bacillus sporogenes*, and *Bacillus bifermentans* were sown. Single spores from a 48 hour culture of *Bacillus subtilis* gave all positives in six tubes of glucose broth with a vaseline layer. The medium was the same as that used for anaerobes except that the free oxygen was not expelled by heat. These results would indicate that the technique of the transfer of the single organism is not at fault, and that failures to grow should be ascribed to a lack of viability of the organism or to its failure to adapt itself to a new environment. The viability of anaerobic bacilli is affected by exposure to air, a fact which explains in part the small proportion of positives as compared with single bacilli or cocci of aerobes. The negatives in spore sowings can hardly be due to lack of proper conditions of anaerobiosis, since spores are little sensitive to short exposures to air, and abundant time is available for the reduction in the new medium of the small quantity of free oxygen introduced with the spore. The quantity of medium to which the spore is transferred can hardly be an important factor in itself, since spores sown in small hanging drops give a smaller proportion of positives than when sown into test-tubes. The quality of the medium is probably more often the determining factor, and if viable spores and a medium exactly suited to each species are employed, all, or practically all the spores might be expected to grow. The process by which an organism adapts itself to a new and less favorable medium is imperfectly known. Our experiments indicate that organisms apparently similar vary greatly in their power of adaptation.

SUMMARY.

The pipette method has proved a feasible method of obtaining one-cell pure cultures of anaerobes.

Both bacilli and spores may be used as seeding material, but spores give a much higher percentage of positives.

Boiling alone affords a sufficient degree of anaerobiosis to the medium for initiating one-cell growths, and semisolid agar is the most convenient form of medium.

Exposure to air during isolation apparently has no effect on the viability of spores of anaerobes, but young bacilli of some species suffer from a comparatively short exposure to free oxygen.

I wish to thank Colonel F. F. Russell, in charge of the Army Medical School at Washington, where a portion of this work was done, and Major Benjamin Jablons, recently in charge of the work on anaerobic bacteria in the Army Medical School, for many courtesies shown and for a large proportion of the cultures of anaerobes used in these experiments. I am also indebted to Dr. Charles Krumwiede, of the Research Laboratories of the Department of Health of the City of New York, for a quantity of tetanus antitoxin.

BIBLIOGRAPHY.

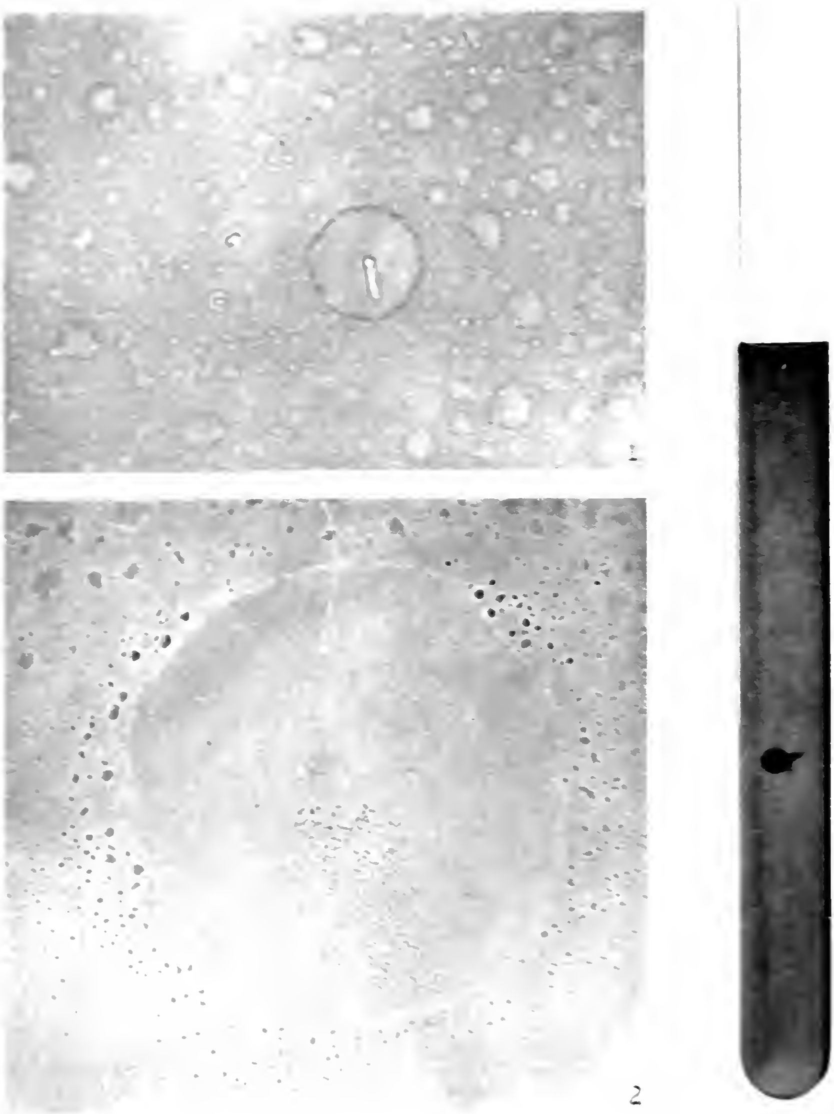
1. Barber, M. A., *Philippine J. Sc.*, 1914, ix, 307.
2. Hort, E. C., *J. Hyg.*, 1920, xviii, 361.
3. Lignières, J., *Compt. rend. Soc. biol.*, 1919, lxxxii, 1091.
4. Bachmann, F., *Centr. Bakteriol., 2te Abt.*, 1912–13, xxxvi, 1.
5. Burri, R., and Kürsteiner, J., *Centr. Bakteriol., 2te Abt.*, 1908, xxi, 289.

EXPLANATION OF PLATE 23.

FIG. 1. Spore-bearing bacillus of *B. tertius* isolated in a hanging drop of broth. × 1,000.

FIG. 2. Colony of *B. sporogenes* grown from a single spore in a hanging drop of semisolid glucose agar. × 120.

FIG. 3. Colony of *B. tetani* grown from a single spore in glucose agar containing tetanus antitoxin. About natural size.



1

2

3

EXPERIMENTAL INOCULATION OF MALARIA BY MEANS OF ANOPHELES LUDLOWI.*

By SAMUEL T. DARLING, M.D.

(*From the Laboratory of the Uncinariasis Commission to the Orient* (*The Rockefeller Foundation*), *District Hospital, Kuala Lumpur, Federated Malay States.*)

(Received for publication, April 14, 1920.)

Doubt has existed in the minds of certain sanitarians in Farther India as to the part played by *Anopheles ludlowi* as a transmitter of the malaria on the littoral of the Peninsula of Malacca and Indonesia.

Christophers in a report of his investigation[1] of malaria in the Andaman Islands in 1912 records the discovery of malarial zygotes in two out of 53 naturally infected wild specimens of *Anopheles ludlowi* and found that the relation between the distribution of the species and that of malaria was most exact.

Watson,[2] however, has expressed the opinion that, from what he has seen in the Federated Malay States, *Anopheles ludlowi* can exist without producing malaria and that malaria will disappear from a place when operations are undertaken which abolish only *Anopheles umbrosus* yet which leave very large numbers of *Anopheles ludlowi.*

Strickland in his report to the Principal Medical Officer of the Federated Malay States, on the cause of the outbreak of malaria at Morib, Federated Malay States, concluded from epidemiological findings that "*Anopheles ludlowi* was causing most of the trouble."

This disparity of opinion among British workers as to the part played by *Anopheles ludlowi* in transmitting malaria is largely if not wholly due to the presence of other species known to be hospitable hosts for malaria, notably *Anopheles umbrosus.* At Port Swettenham, for example, I collected at and near the Rest House *Anopheles ludlowi, Anopheles kochi, Anopheles umbrosus, Anopheles rossi, Anopheles*

* This work was done under the auspices of the British Colonial Office and with the Support of the Government of the Federated Malay States and The Rockefeller Foundation.

[1] Christophers, S. R., Malaria in the Andamans, *Sc. Mem. Med. and San. Dept., Gov. India, No. 56*, 1912.

[2] Watson, M., Rural sanitation in the Tropics, London, 1915, 83.

indefinitus, *Anopheles tessellatus*, *Anopheles barbirostris*, *Anopheles albirostris*, and *Anopheles sinensis*. Barber succeeded in a more exhaustive search in taking one specimen of *Anopheles karwari*. He has also succeeded in infecting all the species mentioned except *Anopheles tessellatus*. Hence the occasion for the doubt that existed in the minds of the local epidemiologists as to the importance of *Anopheles ludlowi*.

In 1916 while studying the cause of anemia among the natives of Java I found epidemiological evidence that the malaria in certain villages, or dessas, near Cheribon on the north shore of Java was due to *Anopheles ludlowi*. In the dessa of Gebongelir there was severe malaria, the splenic index at the time was 97 (101 persons examined), and the endemic index, or parasite rate, was 29 per cent. Mosquitoes were collected daily from the houses of the villagers for dissection. The species chiefly encountered was *Anopheles ludlowi*. Six of the 58 specimens dissected contained malarial zygotes. There were a good many breeding places in the fish ampangs, or reservoirs, near by, and from larvæ collected thereabouts *Anopheles ludlowi* was the only species that emerged. At the same time 89 *Anopheles rossi* Giles from the adjoining village of Gebongoedik were examined for sporozoites and 32 for zygotes with negative result. The weight of the evidence here is that *Anopheles ludlowi* was the carrier of malaria in the village.

It is this epidemiological evidence that is of most value to the sanitarian, for it is possible for *Anopheles* hospitable to malaria to bite malarial patients and become infected and to have sporozoites in their salivary glands, yet if the mosquitoes feed on animals as well as man the infectious sporozoites may often be dissipated on the latter. This is a matter that has been overlooked in discussing the question of efficient agents for transmitting malaria.

Barber[3] has shown that most *Anopheles* in Malaya can be infected with malaria under experimental conditions. Yet I have taken *Anopheles umbrosus* and *tessellatus* which had been feeding on fowl blood for they were caught near hens' nests with nucleated red blood cells in their midguts, and I have also taken many specimens of *Anopheles umbrosus*, *Anopheles rossi*, and *Anopheles indefinitus* in cow barns.

[3] Barber, M. A., Malay *Anopheles* in transmission of malaria, *Philippine J. Sc., Section B*, 1918, xiii, 1.

In Brazil *Anopheles albimanus* feeds with avidity on horses and the latter animals are used as bait to catch the mosquitoes. It is evident that when a species of *Anopheles* known to be a carrier fails to take up an obligate parasitic relation with man and through feeding on animals becomes essentially only a facultative parasite, it cannot be regarded as a thoroughly efficient host for malaria no matter how easily it may become infected in the laboratory. It is the epidemiological evidence, therefore, in the last analysis which convicts a species and will determine the necessity for taking steps against it.

These observations in Java were made in August to October, 1916, at a time when I had already become thoroughly convinced of the importance of *Anopheles ludlowi* as a carrier of malaria in the littoral of Indonesia. The previous year, October to December, 1915, I had found that *Anopheles ludlowi* could be infected experimentally with ease. Out of a number of *Anopheles ludlowi* reared from larvæ taken at Port Swettenham, Federated Malay States, and fed on suitable patients that had sufficient gametes in their peripheral blood, 40 were dissected at intervals of 2 to 12 days after feeding. 20 were found to have zygotes in their midguts and 5 to have sporozoites in their salivary glands. 25 out of 40, therefore, had become infected, a percentage of 62. It was evident that not only could zygotes develop in the tissues of this species but sporozoites could reach the salivary glands and these in a sufficient number of mosquitoes to warrant the assumption that the species was a carrier of malaria. Barber[3] at the same time from a series of experiments came to the same conclusion.

Since then[4] studies on the relation of *Anopheles ludlowi* to malaria in Java and Sumatra have been published by Schueffner, Swellengrebel, Swellengrebel de Graaf, and native Javanese doctors. In these papers *Anopheles ludlowi* is shown conclusively to be a carrier of importance.

While engaged in examining the dissection of the salivary glands it appeared to me that the lumen of the gland ducts was very small, perhaps too small to permit the passage of sporozoites outward from the acini into the proboscis of the mosquito. If this were the case,

[4] Schueffner, W., Swellengrebel, N. H., and Swellengrebel de Graaf, J. M. H., Mededeelingen van den Burgerlijken Geneeskundigen Dienst in Nederlandsch-Indië, 1919, 1.

malarial plasmodia might readily enough develop in the species and sporozoites reach the salivary glands, yet the mosquitoes would be unable effectively to transmit malaria.

Additional antimalarial drainage operations at this time were being planned by the Malarial Advisory Board. It was of some importance for their engineer to know whether measures should be adopted against *Anopheles ludlowi* in addition to those projected against *Anopheles umbrosus* which breed in entirely different locations. For this as well as for other reasons it was decided to undertake inoculation experiments with *Anopheles ludlowi* on man.

EXPERIMENTAL.

Inoculation Experiments.

The experiments were planned to determine the following points: (1) Whether *Anopheles ludlowi* infected under experimental conditions serves to infect man with malaria and with what facility; (2) whether infected mosquitoes that have already bitten one person serve to infect others; (3) the period of incubation in man; (4) the rate of appearance of plasmodia in the inoculated person and how it corresponds with the appearance of fever; (5) what differences if any can be noted in the clinical picture of (*a*) a man being infected with malaria for the first time, (*b*) a man being reinfected with malaria after a long interval of years during which time he has been free from evidences of the disease, (*c*) a man being infected with subtertian malaria who is at the time suffering from tertian malaria; and (6) what type of febrile reaction is induced by a single inoculation of an unmixed strain of subtertian plasmodia (sporozoites).

Malaria is highly endemic in many places outside the cities, towns, and certain protected areas in the Peninsula of Malacca. Several attacks of malaria may be expected by every coolie during his period of residence in the country, particularly if he lives on a rubber estate. Consequently, when the nature of the test was explained to them, there was no difficulty in securing three men who volunteered for the experiment.

316

In all the text-figures the space and dotted lines indicate an interval during which the temperature remained within normal limits.

TEXT-FIG. 1. Temperature curve of Subject 1.

Subject 1 (*Text-Fig. 1*).—L. K. Male, age 37 years; a Chinese mining coolie, resident 18 months in Malaya with a negative history for malaria. He was a patient in the surgical ward with a broken leg; otherwise normal and in good health. Spleen normal; blood negative for malarial plasmodia.

Dec. 1 to 3, 1915, p.m. Bitten by six mosquitoes. Dec. 8. Blood count: Red blood corpuscles 4,829,800; leucocytes 6,200; hemoglobin 85 per cent. Dec. 14. The first clinical note was made on this date when the patient complained of loss of appetite; his tongue was furred. 4 p.m. Temperature 99.4°F. Dec. 15. Pain in back and joints; face flushed; spleen which was normal became palpable below costal margin. 3.30 p.m. Rigors. 6 p.m. Temperature 103.6°F. 9 p.m. Subtertian rings were found in blood. Dec. 16. Sweated a good deal but feels well during the remission of fever. Subtertian rings in blood. Dec. 17, 1 p.m. Mild rigor. Spleen palpable below costal margin. 2 p.m. Temperature 102.2°; tongue coated; bad taste in mouth. Subtertian rings in blood. Dec. 18. Spleen palpable; tender. 4 p.m. Temperature 101.8°F. Subtertian rings in blood. Red blood corpuscles 5,100,000; hemoglobin 79 per cent. Dec. 19. Very ill; vomiting is troublesome. 12 m. Temperature 104°. Spleen 1 fingerbreadth below costal margin; tender. Very few subtertian rings in thin film of blood. Quinine administration begun. Quinine bihydrochloride 15 grains three times a day; retained about 30 grains only. Dec. 20. Very ill; vomited some quinine and all food. Subtertian rings in blood. Dec. 21, a.m. Better; vomiting less frequent; able to retain quinine 20 grains twice a day. Very few subtertian rings in blood. Dec. 22. Very weak; vomiting ceased; 10 grains of quinine three times a day. Blood negative. Dec. 23. No fever; a little better; spleen palpable below costal margin but diminished slightly in size; 10 grains of quinine three times a day. Dec. 24 and 25. Feels better; spleen just palpable; 10 grains of quinine twice a day. Dec. 26. Quinine discontinued. Jan. 1 to 11. There were at times epigastric pain, flushing of the face, and loss of appetite with slight elevations of temperature similar to those observed before the malarial infection. Jan. 17. Patient quite well. Jan. 28. Discharged.

In Subject 1 there was observed a diminution in body weight beginning about Dec. 10 before the onset of fever and lasting until about Jan. 17, 1916 (Table I). On Dec. 22, or the day after the crisis, the loss in weight amounted to 3 catties, or about 4 pounds. His physical strength as measured by the average of three squeezes with the hand grip dynamometer showed a corresponding diminution, dropping from 68.6 on Dec. 11 to 55 on Dec. 24, and returning slowly to 68.6, or normal, 1 month after the crisis.

This was a severe case of malaria with relatively few plasmodia and only moderate enlargement of the spleen.

Subject 2 (*Text-Fig. 2*).—S. Male, age 30 years; Tamil coolie, resident 18 months in Malaya. Has had several attacks of tertian ague with fever and rigor on alternate days during the past 6 months, and on Dec. 1, 1915, tertian plas-

modia of all ages were encountered in the peripheral blood. He did not complain of fever but an inspection of his chart shows quotidian oscillations reaching 99° or 99.5° daily. The spleen was not palpable. He was operated on for hemorrhoids Nov. 11.

Dec. 1. Bitten by six mosquitoes that had a few seconds before been interrupted while feeding on Subject 1. Dec. 8. Blood count: Red blood corpuscles 2,732,800; leucocytes 3,600; hemoglobin 60 per cent. The blood picture from Dec. 1 to 22 consisted in varying numbers of tertian plasmodia. Dec. 12. Tertian rosettes were seen; on the following 3 days there were quotidian rises of tempera-

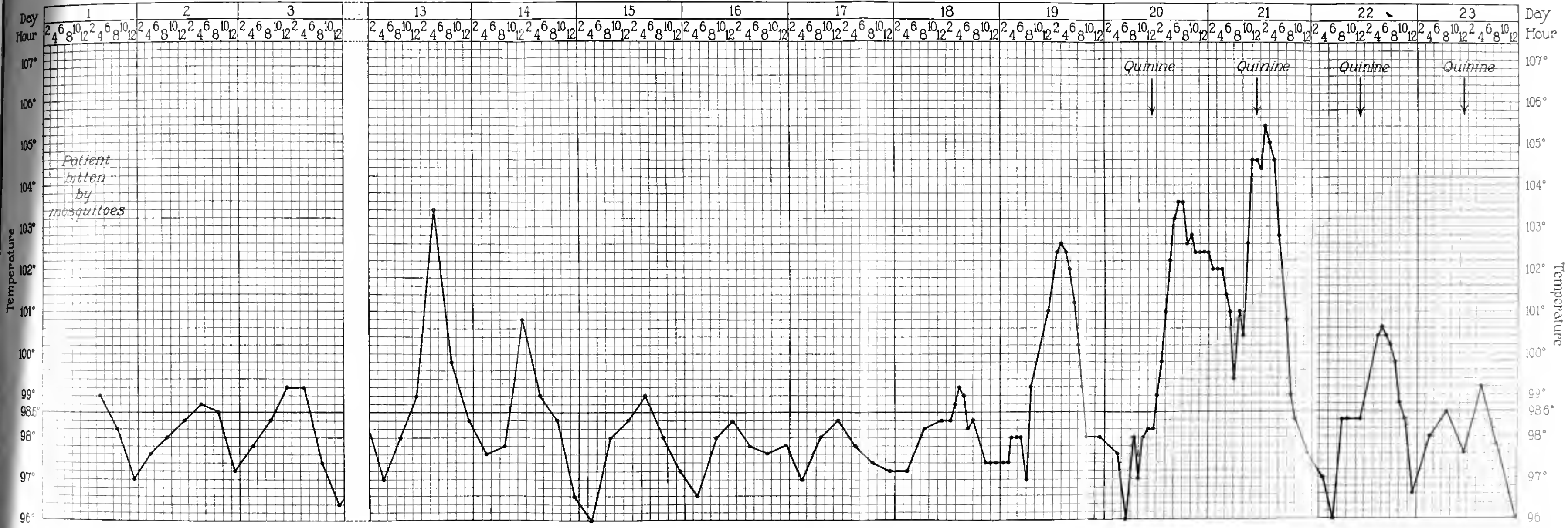
TABLE I.

Body Weight and Strength (Hand Grip) of Subject 1.

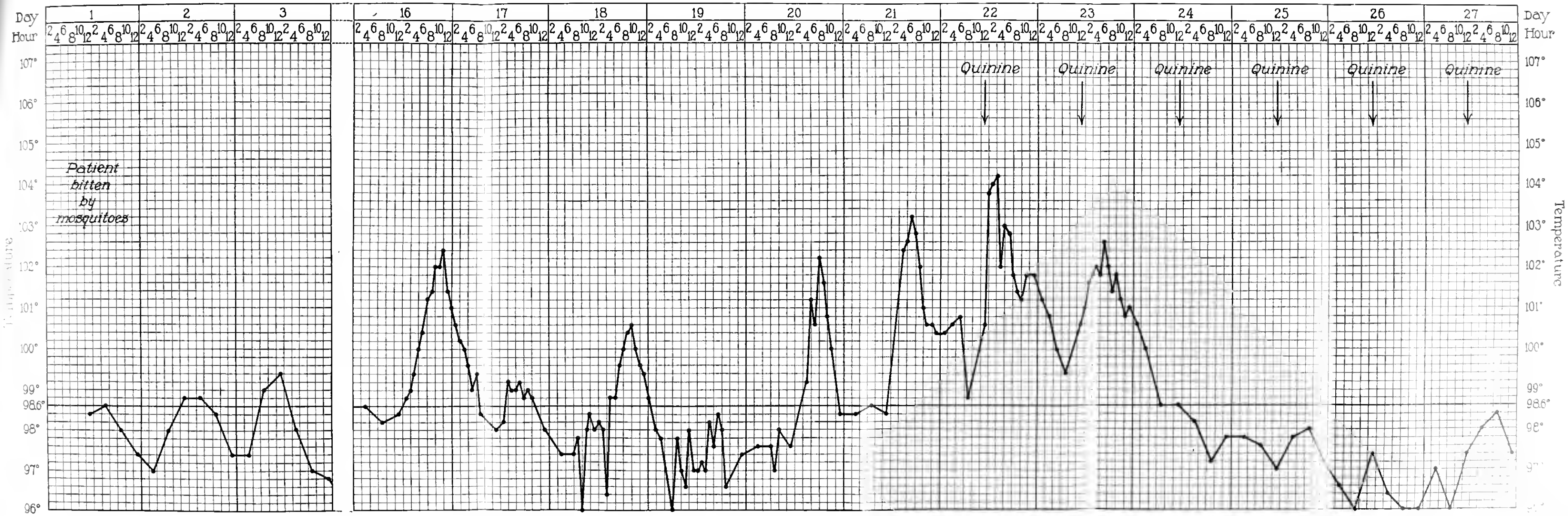
Date.	Hand grip.	Weight in catties.*
1915		
Dec. 6		85
" 8	68.3	
" 10		84
" 11	68.6	
" 16	63	83
" 20	57.3	
" 22		82
" 24	55	
" 28	59	83
1916		
Jan. 1	62.3	
" 3		83
" 4	63.3	
" 9	63.3	
" 10		80
" 13	65	
" 17	65	85
" 20	68.6	85

* A catty is the oriental weight equal to $1\frac{1}{3}$ pounds avoirdupois.

ture. Dec. 13 to 17. A few trophozoites resembling subtertian forms were seen. Dec. 18. Blood count: Red blood corpuscles 3,149,600; hemoglobin 55 per cent. Dec. 20. Subtertian rings definitely present in large numbers. On account of the ease with which some very young tertian trophozoites may be mistaken for subtertian merozoites one can never be absolutely certain of the identity of any given trophozoite. The specific identity of the forms seen on Dec. 13 and 17 cannot be asserted with absolute confidence. Dec. 21. Many subtertian rings were seen. Dec. 22. Fewer subtertian rings were seen. Dec. 23 to Jan 12, 1916. No trophozoites either tertian or subtertian were detected in thick films.



TEXT-FIG. 2. Temperature curve of Subject 2.



TEXT-FIG. 3. Temperature cur e of Subject 3.

Clinical Notes.—Dec. 13. Temperature rose to 104.4°F. without rigor.[5] Apparently he was suffering no inconvenience from this tertian infection. The spleen was not enlarged. Dec. 19, 4 p.m. Temperature rose to 102.4°. Spleen not enlarged. Patient appeared normal. Dec. 20, 2 p.m. Temperature 99.8°. 6 p.m. Temperature 103.6°. Quinine bihydrochloride 10 grains three times a day. Dec. 21, 12 m. Temperature 104.6°. Quinine bihydrochloride 10 grains three times a day. Dec. 22. Patient was slightly inconvenienced by the infection; quinine bihydrochloride 40 grains; spleen not enlarged. Dec. 23 to 29. Quinine bihydrochloride 10 grains three times a day. Patient appeared quite normal, but there was a decided daily drop in temperature from 12 m. to 8 a.m. Dec. 30. Quinine discontinued. Dec. 31 to Jan. 7. Patient quite normal. Weight increased from 100 catties on Dec. 28 to 105 catties on Jan. 17. Jan. 22. 50 hookworms were expelled after a treatment with chenopodium.

This was a mild case of malaria as far as symptoms were concerned, but the number of plasmodia encountered was considerable. The febrile reaction, size of spleen, and multiplication of subtertian plasmodia were not in the slightest degree influenced by or interfered with by the preexisting tertian infection.

Subject 3 (*Text-Fig. 3*).—J. Male, age 45 years; a Tamilian hospital attendant, resident 26 years in Malaya. During the past 7 years he had been entirely free from attacks of malaria but previous to this he had suffered from malaria several times, the attacks lasting about 10 days. Was being treated for a compound fracture of ulna with necrosis of bone. Blood was negative for malaria. Spleen normal.

On Dec. 3, 1915, bitten by three mosquitoes and on Dec. 4 by two mosquitoes that had previously fed on Patients 1 and 2 on Dec. 1. Dec. 8. Blood count: Red blood corpuscles 3,680,000; leucocytes 7,600; hemoglobin 57 per cent. This anemia may have been related to the injury to his elbow. There was certainly no malaria and no ancylostomiasis. Dec. 17. Facial expression has changed; patient looks drowsy. Complains of pain in elbow. The spleen which had been negative is palpable below the ribs. Dec. 18. Appeared well. Very few subtertian rings in thick film of blood; thin film negative. Red blood corpuscles 3,949,600; hemoglobin 66 per cent. 8 p.m. Temperature 101.4°, with rigors. Dec. 19. Spleen 2 fingerbreadths below ribs and tender; fever and rigors previous night; not very ill. Subtertian rings in thick film of blood; thin film negative. Dec. 20. Fever and rigor. 8 p.m. Temperature 100.4°. Spleen unchanged;

[5] The occurrence of latent malaria without symptoms is far more common than is suspected. In Panama I found 10 per cent of 322 Spanish laborers to be harboring plasmodia in their blood. The blood specimens were taken at the mess hall and none of the men was ill or complaining at the time.

not very sick. Thin film of blood negative. Dec. 21. Marked remission; spleen unchanged. Few subtertian rings in thick film of blood. Dec. 22. Rigors and fever. 8 p.m. Temperature 101.8°. Spleen 2½ fingerbreadths below ribs; conjunctivæ injected; face flushed. 11 p.m. Sweating. Fewer subtertian rings than yesterday in thick film of blood. Dec. 23. Condition the same. 4 p.m. Temperature 102.4°. Fewer subtertian rings in thick blood film than day before. Dec. 24. Very ill and weak. 4 p.m. Temperature 102°. Spleen at level of umbilicus and tender; tongue coated. Dec. 24 to Jan. 11. Blood negative. 8 p.m. Quinine bihydrochloride 20 grains. Dec. 25. Very weak. Temperature 102°. Quinine bihydrochloride 15 grains three times a day. Dec. 26. Better; temperature normal; spleen 1 fingerbreadth below umbilicus; tender; no vomiting. Dec. 27. Quite well. Dec. 28. Quinine bihydrochloride 10 grains three times a day. Jan. 8, 1916. Quinine discontinued. Jan. 11. Spleen at level of umbilicus. Jan. 18. Spleen 2 fingerbreadths above level of umbilicus. Jan. 20. Discharged.

This was a case of malaria of ordinary degree of severity in which plasmodia were not very abundant but in which the splenic reaction was pronounced and was manifest before plasmodia appeared in the peripheral blood.

Method of Infecting Mosquitoes and Inoculating the Men.

The method was identical with that used in Panama[6] in 1908. Full grown larvæ of *Anopheles ludlowi* were collected at Port Swettenham and reared at Kuala Lumpur. The mosquitoes after emerging were confined in biting jars made of lantern chimneys. The air in the jars was kept as moist as possible and sugar was given when necessary. Both ends of the jars were securely covered with crinoline gauze through which the mosquitoes bit. The mosquitoes were at first applied daily to the skin of a gamete carrier in order to favor the infection of as many mosquitoes as possible and also to reduce the mortality from desiccation. The jar of mosquitoes used was comprised of adults that had emerged from pupæ Nov. 12, 1915. They were fed as shown in Table II. After Nov. 20 they were fed on sugar alone. Nov. 21. Four mosquitoes were dissected. One contained five zygotes 19μ in diameter. Nov. 25. Five mosquitoes were dissected. Two contained zygotes; one contained one zygote, the other seven. Nov. 26. Dissected six mosquitoes. Two contained zygotes; one had one large zygote, the other seven or eight very large zygotes. One of these zygotes displayed sporoblasts; three of the other zygotes contained sporozoites. Nov. 28. Dissected four mosquitoes. One contained two large zygotes with sporozoites, another displayed three large zygotes with sporozoites.

[6] Darling, S. T., Studies in relation to malaria, Panama, Canal Zone, Gatun I. C. C. Press, 1916.

Thus out of nineteen mosquitoes that survived, seven, or 37 per cent, were positively infected. The jar still contained seven mosquitoes two or three of which were probably infected. The seven survivors were used in the inoculation experiments on Dec. 1, 3, and 4. On Dec. 6 the three that were left were killed and dissected. Two of the three contained a few sporozoites in the salivary glands and ducts.

Biting Experiment.—Dec. 1, 1915, 3 p.m. The jar containing infected *Anopheles ludlowi* was applied to Subject 1. Six of the seven mosquitoes fed. The feeding was interrupted and the same jar was now applied to Subject 2. Six mosquitoes were seen to feed. Judging from the degree of distension of their abdomens apparently they were the same ones that had just before fed on Subject 1. Dec. 3. The jar was applied to Subject 3, and three mosquitoes fed. Dec. 4. The jar was again applied to this patient and two mosquitoes fed.

TABLE II.

Time of feeding.	Patient No.	Gametes present in patient's blood.
1915		*per cent*
Nov. 16, a.m. and p.m.	4	3.9
" 17, p.m.	4	3.1
" 18, "	4	2.4
" 19, "	5	33.6
" 20, "	5	33.3

In none of the dissections of infected *Anopheles ludlowi* were zygotes ever encountered that showed signs of degeneration or delayed development. The species therefore offers a favorable medium for this plasmodium and is evidently an efficient host. As from 40 to 60 per cent of the mosquitoes were found to have become infected, we are safe in assuming that among the mosquitoes that bit the patients about half were infected and contained sporozoites. As a matter of fact, two of the three survivors at the conclusion of the experiment actually had sporozoites in their salivary glands. There is no certitude, however, as to the actual number of infected mosquitoes that bit the patients. Subjects 1 and 2 were certainly bitten by one and possibly by two or three infected mosquitoes. Subject 3 was bitten by one or possibly by two infected mosquitoes. Since two infected mosquitoes with sporozoites in their ducts were found after the conclusion of the biting experiments, it is likely that each man was bitten by at least two infected mosquitoes. They were certainly bitten by one, for each patient gave clinical evidence of infection.

Cases from Which the Mosquitoes Were Infected.

Dissections of mosquitoes in this experiment and others not recorded here indicate that the mosquitoes were infected from Subject 4 and not from No. 5. This belief is based on the failure to find small zygotes corresponding in size with those which should have been derived from No. 5. Every carrier of gametes is not infectious for mosquitoes. I pointed this out in 1908 and Dr. Barber and I have confirmed the fact several times since. In selecting a gamete carrier for incrimination experiments one with a sufficient number of gametes is necessary. From observations made in Panama in 1908 it would seem to me that at least twelve gametes per c.mm., or one for every 500 leucocytes, are necessary.

Infectivity of Anopheles ludlowi Infected under Experimental Conditions.

It can be asserted that when a gamete carrier has as few as 2.4 to 3.9 gametes per 100 leucocytes about 40 per cent of *Anopheles ludlowi* may be infected from him. These mosquitoes may have from one to eight zygotes and will furnish a sufficient number of sporozoites to infect man. The size of the salivary ducts offers no impediment to the free exit of sporozoites, and inoculation occurs with ease either in an interrupted feeding or after two previous feedings.

In view of these experiments and the positive epidemiological evidence advanced above *Anopheles ludlowi* must be regarded as one of the important carriers of malaria and must be included in the list of *Anopheles* against which antimalarial measures must be taken.

Power of Infected Mosquitoes That Have Already Bitten and Infected Man to Infect Others.

In an interrupted feeding mosquitoes first bit and inoculated Subject 1 and a few seconds later the same mosquitoes bit and infected Subject 2. 2 and 3 days later two to five of these mosquitoes fed on and inoculated Subject 3 with sporozoites without any demonstrable diminution of infecting material as measured by the symptoms. Moreover, surviving mosquitoes were found to contain sporozoites in their salivary glands.

We may assert that infected *Anopheles* may retain infection after at least three bitings. The biting habits and feeding exigencies of *Anopheles* are not thoroughly understood. That is, we do not know how many times an *Anopheles* may feed or how many times it is accustomed to feed between receipt and transference of infection. In the laboratory they may be induced to bite every other day. In nature they return to the breeding place after the blood meal has been digested. More or less lengthy flights are often necessary and feedings may occur only in response to demands for ovulation. The circumstances indicate that an *Anopheles* may remain infected throughout life and may infect at each feeding.

Period of Incubation.

The period of incubation as measured from the date of inoculation to the occurrence of fever was 14 days in Subject 1, and 15 days in Subject 3 if counted from December 3, the date of the first biting by three mosquitoes, or 14 days if counted from the date of the second biting, December 4. In Subject 2 the period of incubation was 18 days. This patient was already suffering from a tertian infection and the blood picture on December 12 and 13 indicated that the rises of temperature on December 13 and 14 were tertian paroxysms and not due to subtertian plasmodia. Subtertian malaria is not frankly exhibited until December 19 to 21, the access of fever coming on December 19 and many subtertian rings appearing in the blood on December 20. The period of incubation, therefore, was 18 days.

Rate of Appearance of Plasmodia in the Peripheral Blood and Its Correlation with Fever.

The rate of appearance of plasmodia in the peripheral blood will depend somewhat on the method of examination; thick films consisting of larger quantities of blood will disclose plasmodia earlier and in greater numbers than thin films. In Patient 3 on December 19 a thin film was negative, while the thick film was positive for plasmodia. A thick film represents about 1 c.mm. of blood. When plasmodia have become sufficiently numerous to be found in a film of this size they exist in large enough numbers to cause a paroxysm of fever. This was observed in all three cases.

Differences in the Clinical Picture and in the Spleen Reaction under Different Conditions.

Individual Infected for the First Time with Malaria.—Patient 1, infected for the first time, may have received a larger dose of sporozoites than the others. His blood, however, never disclosed an unusually large number of plasmodia, and his symptoms were rather disproportionately severe in relation to the number of plasmodia found. We know from abundant observations the truth of Manson's statements that the internal viscera are the organs involved in malaria and that the plasmodia in the peripheral blood often represent an overflow and are not an exact expression of the number of plasmodia actually present in the body. It may be asserted that the symptoms were more severe in this case of primary infection. The splenic enlargement began to manifest itself by tenderness on the 1st day of fever and at a time when plasmodia were first discovered in thick films. The spleen reached 1 fingerbreadth below the costal margin on December 19, or 4 days after the onset of fever. It returned almost to normal proportions December 24. In this case there is a correspondence in time between the appearance of splenic enlargement, the appearance of plasmodia in thick films, and the onset of fever, all of which occurred on or about the same day. The spleen remained enlarged to palpation only about 11 days.

Individual Infected after a Long Interval of Freedom from Malaria.—Patient 3 was the third case to be bitten and he may have received a smaller dose than the patient previously bitten. We note that the period of incubation was 15 or 14 days, or about the same as that of Case 1. The appearance of plasmodia in thick films on December 18 was accompanied by a rise in temperature, but the patient while showing evidences of infection was not really ill until December 25, or the 8th day of fever, whereas Patient 1 was acutely ill on the 5th day. No. 3 had a pronounced splenic reaction. The spleen which had been negative upon palpation was palpable just below the costal margin on December 17, or the day before the initial rise in temperature. It reached its maximum size 1 fingerbreadth below the umbilicus on December 26, the day that the temperature reached normal after the last rise in temperature, or 8 days after the initial rise in

temperature. Nearly the same degree of enlargement was present for several weeks later, for on January 18 it was still 1 inch above the umbilicus. Here we note a prompt reaction and prolonged period of enlargement, the degree of enlargement being that of splenomegaly and occurring in a man who had previously suffered from several attacks of malaria.

Individual Already Suffering from Tertian Malaria.—Patient 2 not only appeared to experience no inconvenience from the tertian infection from which he was suffering, but the additional infection of subtertian malaria likewise elicited no evidence of physical illness. The dose in this case can be assumed to have been at least as great as in Case 3 for he was the second man to be bitten. The period of incubation was 18 days. It is possible that the coexisting tertian infection influenced the duration of the period of incubation. Subtertian plasmodia appeared in large numbers on December 20 and in notable numbers before and after this date as well as could be ascertained. It is certain, therefore, that no immunity reaction had been provoked by the tertian infection which would tend to prevent the development of the superimposed subtertian infection. The spleen showed no response to either infection by tenderness or enlargement.

Type of Febrile Reaction and the Probable Invalidity of Plasmodium falciparum quotidianum as a Species Distinct from Plasmodium falciparum.

Text-figs. 1 to 3 not only display the type of fever seen during the period of incubation and access of fever, and the effect of quinine in reducing the fever, but they are of unusual interest in exhibiting the peculiar type of febrile reaction elicited in persons experimentally infected with a pure strain of subtertian plasmodium. The types of temperature graphs seen in clinical cases of malaria that have run for longer or shorter periods are often the result of multiple or superimposed infections. The phenomenon of acceleration or retardation of paroxysms often causes irregularly continuous curves. In malarial communities in the Tropics mixed infections are extremely common, and it is often difficult to diagnose the type of plasmodium by an inspection of the chart. Our knowledge of the types or species of ma-

larial plasmodia has been almost wholly advanced by studying the disease in persons who have been infected naturally, in whom the disease has run for a longer or shorter period of time, and in whom acceleration and multiple infection have complicated the clinical picture.

The study of the morphology of plasmodia is beset with difficulties inherent in a science which is limited to studies of form and staining reactions. There is usually little difficulty in differentiating most of the forms of plasmodia of quartan, tertian, and subtertian malaria. Young trophozoites and young gametes at times cause trouble. It is easy enough to follow the drawings of the text-books and assert because of resemblances of forms in the slide to pictured representation of what authors have chosen to call young tertian trophozoites or malignant tertian gametes that the former actually are identical with the latter, but whether they are absolutely so or not is an entirely different matter. For example, in the cases here described all the infections resulted from a case of malaria the blood of which contained no other forms but crescents. No tertian plasmodia were ever seen, yet when the experimental cases began to reveal plasmodia in their blood there were slight differences noticeable in some of the young trophozoites. When, for purposes of demonstrating the personal equation in the specific identification of plasmodia, these were shown to experienced microscopists, some trophozoites were called subtertian, others benign tertian. As none of these young "tertian" trophozoites ever matured into more adult forms, the assumption was that they were all subtertian as they should have been, and the microscopist was mistaken in his diagnosis as he undoubtedly is occasionally.

Text-books contain references to two kinds of *Plasmodium falciparum*; namely, a tertian and a quotidian variety. This is largely due to the accounts of two varieties of fever and plasmodia by Marchiafava and Bignami. Mannaberg, Manson, Grassi, Feletti, and Craig are of this opinion also. According to Marchiafava and Bignami, the quotidian variety causes a paroxysm every 24 hours. The fever may be regular like the benign tertian, but oftener exhibits acceleration or retardation, becoming irregularly remittent or continued.

The malignant tertian plasmodium of Marchiafava and Bignami resembles the quotidian very closely but according to them is larger and contains a greater amount of pigment. It may become one-half the size of an erythrocyte. The malignant tertian plasmodium possesses greater ameboid activity. The quotidian plasmodium is very pale; it never exceeds one-third the size of an erythrocyte. The infected erythrocyte has a brassy color, is often shrunken, and its hemoglobin is retracted.[7]

An inspection of the temperature charts of the two uncomplicated cases, Nos. 1 and 3, will show that before there has been any administration of quinine the initial paroxysms are tertian or subtertian in periodicity and that the subsequent ones are quotidian. Moreover, in each intermission of fever in the tertian phase there is a slight rise of temperature about 4 p.m., thus giving evidence of a latent quotidian tendency even in the tertian phase which soon manifests itself as a distinct quotidian wave which may be followed by a remission or definite intermission of fever. Thus we have the interesting phenomenon of a pure strain of subtertian plasmodia provoking in infected persons a febrile reaction which at first is distinctly tertian in periodicity. This is immediately succeeded by a reaction having quotidian periodicities.

If we accept the claim of Marchiafava and Bignami, and Craig that there exist two distinct forms of *Plasmodium falciparum*, the quotidian and the malignant tertian varieties, we may account for the phenomenon by assuming that the patient from which the mosquitoes were infected was suffering from both forms of the disease and that the gametes consisted in part of quotidiana and in part of tertiana maligna. But in that case it is difficult to understand why the fever did not manifest itself from the start in more pronounced quotidian form. This theory will not explain the well known tendencies of subtertian infection to exhibit irregularly remittent and continued forms of fever.

We may assume, on the other hand, that there is but one form and species of *Plasmodium falciparum* and that this species possesses inherently both subtertian and quotidian tendencies manifested in

[7] Craig, C. F., The malarial fevers, New York, 1909, 34.

types of fever at times having tertian and at times quotidian periodicities, and also possesses the well known tendencies to cause fever of an irregularly remittent or continued type. This assumption I think is in better agreement with the observed facts. We do not know the cause of the lack of uniform periodicity in *Plasmodium falciparum*, but it may be related to the following facts. Quartan plasmodia sporulate in the peripheral blood and have from 5 to 11 merozoites. In Panama the number was 5 or 6 and very uniform. In Malacca the number was near 11 and also uniform. Tertian plasmodia also show considerable uniformity in the number of merozoites possessed by segmenting plasmodia. In subtertian malaria, however, films from the spleen, marrow, and brain display a lack of uniformity in the number of merozoites, for they have been found to run from 10 or 12 to 42. This morphological heterogony may and probably does in some way correspond to the clinical polymorphism observed in malignant tertian malaria. From my experience in Panama and Malacca it has never seemed to me that there existed sufficiently cogent reasons for creating a specific plasmodium for clinical forms of quotidian æstivo-autumnal malaria.

The diagnosis of *Plasmodium falciparum quotidianum* was oftenest made during the period when fresh films only were examined and before the Romanowsky stains came into use, and the creation of a separate species probably rests on insecure and insufficient testimony. At any rate, the matter requires further elucidation by means of pure strains and inoculation experiments.

SUMMARY.

Three persons were experimentally inoculated with malaria by means of *Anopheles ludlowi* reared from larvæ and infected with a pure strain of subtertian plasmodium (*Plasmodium falciparum*), thus proving that there exists no mechanical impediment or obstacle to the free exit of sporozoites from the salivary ducts or proboscis.

In the dissection of infected mosquitoes there were no evidences of degenerated zygotes. Sporozoites appeared promptly in the salivary glands (9 to 12 days). Inoculation occurred with ease either in an interrupted feeding or after mosquitoes had been fed twice previously.

The period of incubation was 14 and 18 days. The clinical manifestations were more severe in the subject that had never been infected with malaria previously, while the splenic enlargement was most pronounced in the subject infected after a long interval of freedom from malaria. In a third subject already suffering from tertian malaria there was only the slightest evidence of physical illness elicited by the superimposed subtertian infection; his temperature, however, became duly elevated.

The type of febrile reaction in the two uncomplicated cases was at first tertian, becoming quotidian later, and this phenomenon in a pure strain leads strongly to the supposition that *Plasmodium falciparum* possesses inherently both tertian (or subtertian) and quotidian tendencies, as well as its well known tendencies to cause fever of the irregularly remittent or continued type.

The creation of a specific plasmodium to account for clinical forms of æstivo-autumnal or subtertian malaria having a quotidian periodicity is probably unwarranted.

In consideration of the facility with which this species can be infected and man inoculated experimentally, the occurrence of naturally infected wild specimens, and the positive epidemiological evidence, there should no longer exist in the minds of sanitarians any doubt as to its being a malarial carrier.

Operations against this species can therefore be recommended without reservation and should be carried out without delay.

I am indebted to Assistant Surgeon A. E. Durasamy and to my laboratory assistant Mr. Chong Yoon Chong for assistance in obtaining histories and recording clinical notes of the patients.

STUDIES ON BACILLUS MURISEPTICUS, OR THE ROTLAUF BACILLUS, ISOLATED FROM SWINE IN THE UNITED STATES.

By CARL TENBROECK, M.D.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

PLATE 24.

(Received for publication, May 21, 1920.)

No essential differences have been detected between the mouse septicemia bacillus described by Koch (1) and the rotlauf or swine erysipelas bacillus subsequently discovered by Loeffler (2). The studies to be reported here are on organisms obtained from swine which, if they had been isolated in Europe, would undoubtedly have been called rotlauf bacilli, but since swine erysipelas is a disease that has not thus far been recognized in the United States, and since Koch's organism was the first to be described, they are called mouse septicemia bacilli, or *Bacillus murisepticus*.

There are reports that organisms of this group have been isolated three times in this country. Smith (3) in 1885 obtained a culture from the spleen of a pig dead of hog-cholera, and Moore (4) in 1888 obtained a culture from the spleen of another pig affected with the same disease. Smith (5) in 1894 again obtained a culture from the cervical lymph node of a pig that presumably had hog-cholera. This last paper is of special interest, for in it we find the first description of scurvy in guinea pigs and a demonstration that such animals may be susceptible to organisms to which the normal guinea pig is immune. In addition, there is a discussion of the relation of feeding to disease, a subject about which even today we know very little, but the importance of which we are beginning to realize.

These organisms are not difficult to isolate, and if they were at all common in the bodies of pigs with hog-cholera they would have been reported more frequently. In Europe, and especially in Germany, there have appeared many reports concerning their presence in swine. Koch (1) obtained the mouse septicemia bacillus from mice that he had inoculated with putrid material. How many times he found it and how frequently it has subsequently been obtained in this way cannot be determined from the literature. In 1882 Loeffler (2) found similar organisms in the bodies of swine affected with rotlauf, rouget, or swine

erysipelas. They were present in great numbers not only in lesions of the skin but generally distributed in the body, and he regarded them as the cause of the disease. Pasteur and Thuillier (6) had previously studied rouget and described as the cause an organism which from their description is difficult to identify. That they cultivated the organism described by Loeffler is shown by the observations of Schütz (7) and Smith (8), both of whom found the mouse septicemia bacillus in the vaccine prepared by Pasteur for protecting against rouget. The organisms found by Loeffler resembled Koch's bacillus in their morphology, growth in gelatin, and in being virulent for pigeons, white and gray mice, but not for field mice. Olt (9) quotes Loeffler as finding that animals which had recovered from an infection produced by the injection of rotlauf bacilli were immune to *B. murisepticus* and *vice versa*. This power of one organism to immunize against the other has repeatedly been confirmed, and Rosenbach (10) and others have shown that rotlauf serum will protect against the mouse septicemia bacilli as well as against the organisms obtained from swine.

Rosenbach (10) and Preisz (11) found the mouse septicemia bacillus to be slightly larger and to grow more rapidly in gelatin than the organisms obtained from swine. Rickmann (12), on the other hand, studied over 100 cultures obtained from swine with rotlauf and found considerable variation in their morphology, so that he could not differentiate some of them from the one culture of mouse septicemia he had, which was the one used by Rosenbach. Stickdorn (13) found that the frequent transfer of a virulent rotlauf culture or its passage through a series of mice or pigeons changed not only the virulence but the character of the growth in gelatin, and Smith (5) found that the same cultural character was markedly influenced by the reaction of the medium.

When injected immediately after its isolation the rotlauf bacillus may be virulent for swine, but after it has been on culture media for any length of time or after it has been passed through some of the experimental animals it loses this virulence and then resembles *B. murisepticus* in that it fails to cause disease when injected into pigs.

Organisms that could not be differentiated from those found in swine with rotlauf have been isolated from chickens by Schipp (14) and from sheep with arthritis by Poels (15). In continental Europe bacilli of swine erysipelas have also been found in swine that were apparently normal. Bauermeister (16) inoculated fifteen mice with the secretion from the tonsils of fourteen pigs. From five of the mice he obtained cultures of what he called rotlauf bacilli. Olt (9) also found these organisms in normal swine, but he does not give the number of animals examined or the number of cases that were positive. Van Velzen (17) studied the tonsils of eleven normal pigs and from three obtained rotlauf bacilli. Pitt (18) inoculated mice with the secretion from the tonsils of 50 pigs and with the secretion from the glands at the ileocecal valve of 66 pigs. 56 per cent of these swine showed rotlauf bacilli in the tonsils and 40 per cent in the glands at the ileocecal valve.

That these organisms are the cause of rotlauf seems to be generally accepted. The fact that they are usually present in great numbers and the encouraging results obtained by vaccination are in favor of this view. It is, however, unusual to find such a large percentage of normal animals that are carriers of organisms that are the primary agent of a disease. Olt (9) has suggested that these bacteria may gain entrance to the body through lesions produced by intestinal parasites and then become virulent; Smith (5) emphasizes the relation of feeding to an invasion of the body. There is, furthermore, the possibility that the organisms found in normal swine differ from those found in rotlauf. Proof of any of these hypotheses is lacking and the facts as we now know them suggest that these organisms may be secondary invaders. There are diseases in which secondary invaders are almost invariably present and at times, due to their number or the ease with which they are isolated, are regarded as the primary agents of a disease. In some cases it is only by the most careful work and the use of special methods that the primary agent of the disease is recognized. The object of this paper is to call attention to the fact that these organisms occur in the United States where the disease of swine erysipelas is unknown, to give the cultural reactions of the organisms isolated, to record some observations on the pathology of the disease produced in experimental animals, especially the mouse, and finally to raise the question as to whether these bacilli really are the primary agent of rotlauf in swine.

Isolation of Cultures from Swine.

In a study of the tonsils of pigs that had been inoculated with hog-cholera virus some of the exudate from an ulcer on the tonsil was injected into mice. These mice died in a few days and from them were isolated organisms that corresponded to the mouse septicemia bacillus. Following this first observation, material from the tonsils of all the swine that came to autopsy was injected into mice, and when these animals died, cultures were made from the spleen and heart's blood and films from the lungs were stained by Gram's method and examined for the intracellular Gram-positive rods, which are characteristic of *Bacillus murisepticus*.

Sixteen swine, all infected with hog-cholera virus, have been examined in this way and from the tonsils of five of these bacilli have been isolated which morphologically and culturally are mouse septicemia bacilli. The organisms were apparently localized in the tonsils, for they were not found in cultures made by transferring pea-sized bits of liver, spleen, and kidney to agar slants.

Four of the positive swine were from the same litter and the source of the fifth one is questionable, so that it appears that the infection is restricted. On the farm from which these animals came there is no record of any diseases among pigs, and before inoculation the animals were apparently well.

These organisms may have some relation to the ulcers that are so commonly present on the tonsils of pigs with hog-cholera, as four of the five positive cases showed ulcers, whereas only four of eleven negative cases showed them. It is just as possible, however, that a variety of organisms may be associated with these ulcers, as they are probably due to invasion by bacteria of a lesion produced by the hog-cholera virus. Bacilli of the swine-plague group were often present and killed the inoculated mice before the mouse septicemia bacilli had time to invade the bodies of these animals. It would be of interest to know how common and how widespread the mouse septicemia bacilli are in the United States, and if such a study is made it would be well to immunize part of the mice used for their isolation by the injection of anti-swine-plague serum as was done by Van Velzen (17).

Cultural Characters.

The ease with which mouse septicemia bacilli are identified probably accounts for the meager description of their cultural reactions. The morphology has been well described, as has the growth on agar and in gelatin, but this is as far as most descriptions go. It therefore seemed worth while to gather together the recorded reactions and to fill in the most obvious gaps. The five cultures isolated recently and the one culture isolated by Smith (5) in 1894 have been used, and all have been found to give the same results.

Morphology.—These organisms are non-motile, Gram-positive rods, varying considerably in length and diameter, the variation depending

upon the source from which preparations are made. In films from mice, dead after inoculation with a pure culture, they appear as very slender, straight or slightly curved rods from 1.5 to 2 μ in length. They may appear free or are grouped in a characteristic manner in large cells with an indefinite nucleus. From the surface of Loeffler serum slants and in gelatin incubated at 37°C. they appear as straight or slightly curved rods somewhat thicker than those found in the tissues of mice. In bouillon, and especially on agar slants, this increase in size is more marked. The organisms may measure up to 4 μ in length and show a decided tendency to curve and form clumps of interlacing bacilli. Branching forms have not been found in films made from cultures on a variety of media incubated up to 5 days. In sections of tissues stained by the Gram-Weigert method the organisms often have a beaded appearance, but in films stained by Gram's method, using Stirling's gentian violet and decolorizing with alcohol, the organisms stain uniformly.

Agar.—Growth appears in 24 hours in the form of fine, translucent, slightly gray colonies. It is very scanty, but becomes more abundant if serum or defibrinated blood is added to the medium.

Gelatin.—Stab cultures show the characteristic "test-tube brush" growth described by Loeffler (19) which has been serviceable in identifying these organisms. Petri and Maassen (20) obtained the same type of growth in a semisolid medium at incubator temperatures, but I have been unable to confirm this. Smith (5) noted that when the gelatin was acid this characteristic growth did not occur. After some weeks incubation gelatin stabs show a finger-like depression which is not found in uninoculated tubes that are incubated the same length of time. If this is due to a softening and evaporation of the gelatin, as has been stated, it is evident that it proceeds at a very slow rate and that it only occurs in the presence of oxygen. Gelatin cultures that have been incubated at 37°C. for 30 days will still harden when placed in the refrigerator.

Bouillon.—In 24 hours there is a uniform turbidity and when shaken a characteristic cloud-like effect is produced that has been considered of diagnostic value.

Blood Serum.—Several writers state that these organisms do not grow on this medium, while Poels (15) states that it grows but does

not liquefy the serum, this being one of the differential points between the rotlauf bacillus and *Bacillus pyogenes*. All of my cultures have formed a scanty growth on Loeffler serum slants and after 30 days incubation there was no evidence of liquefaction.

Milk.—All writers agree that no visible change is produced in this medium. Moore (4) found microscopic evidence of vigorous growth of the one strain he studied, while Schipp (14) found no evidence of growth of the three strains with which he worked. All of my cultures have failed to produce acid in milk, yet all of them show microscopic evidence of growth. As will be noted below, these organisms attack lactose in bouillon, but not in milk.

Ammonia.—6 day cultures in fermented bouillon failed to show any appreciable amount of ammonia.

Hydrogen Sulfide.—Petri and Maassen (20) found this gas in their cultures, and in swine dying of rotlauf they found sulfmethemoglobin, but they did not find sulfmethemoglobin in experimental animals inoculated with pure cultures. All six of my cultures blackened lead acetate in peptone agar in 24 hours.

Indole.—Schipp (14) has the only reference that I have found to indole production, his three cultures all giving a negative test. All six of my strains in fermented bouillon cultures 5 days old gave a negative test with Ehrlich's aldehyde as did a typhoid culture and the uninoculated medium. A colon culture gave a positive reaction.

Phenol.—No reference has been found on phenol production. Three of my cultures were grown for 10 days, each in 40 cc. of fermented bouillon contained in 100 cc. Erlenmeyer flasks. They were then distilled with steam and the distillate was tested with bromine water and Millon's reagent. Reactions for phenol were not obtained.

Hemolysins.—Van Nederveen (21) found that his culture did not hemolyze swine blood agar in plates, or the blood of cattle, rabbits, or pigeons in bouillon. The six cultures of the present study all produced a distinct zone of hemolysis around the deeper colonies in veal infusion agar plates containing about 10 per cent of sterile defibrinated horse blood. The same blood in bouillon was not hemolyzed.

Fermentation of Carbohydrates.—The literature gives very little information about this important phase of the bacterial activity of these organisms, and that found is not in agreement. Fermi (22) states that

the rotlauf bacillus forms acid from starch but that it does not possess a diastatic enzyme. Petri and Maassen (20) found that the growth of their rotlauf cultures was increased when dextrose, lactose, saccharose, or dextrin was added to the medium. Smith (5) studied one culture and found that acid but no gas was formed in bouillon containing dextrose or lactose, while no acid was formed in bouillon containing saccharose. Schipp (14) studied three cultures and found that acid was formed in a litmus peptone solution containing lactose or saccharose, while in the same medium containing dextrose the litmus was decolorized but was not reddened.

Preliminary tests with fermentation tubes containing fermented bouillon plus 1 per cent of either dextrose, lactose, or saccharose

TABLE I.

Carbohydrate.	Initial reaction.		Length of incubation.	Final reaction.		
	Acidity.	pH		Acidity.		pH
	per cent		*days*	*per cent*		
Dextrose	0.7	7.5	5	2.7–3.1		6.1–5.8
Lactose	0.9	7.5	7	2.4–3.1		6.4–6.0
Arabinose	1.3	7.3	7	1.6–1.8		6.7–6.6
"	1.3		10	1.8–2.1		
" fermentation tubes	1.1		7	Bulb. 1.7–2.1	Branch. 1.1–1.5	

showed that these organisms did not form gas, but produced acid from the first two carbohydrates and that somewhat more acid was produced in the presence of oxygen than in its absence. In saccharose bouillon there was no acid or alkali formed. The great majority of the tests has been made in ordinary test-tubes containing 13.5 cc. of fermented bouillon to which 1.5 cc. of a previously autoclaved 10 per cent solution of the carbohydrate in distilled water was added. The tubes were steamed and incubated to insure sterility and were then inoculated from young bouillon cultures and titrated after from 5 to 7 days incubation. When acid was produced hydrogen ion determinations were also made, but when the carbohydrate was not acted upon this was not done. In Table I are given the extremes of reaction produced by the six cultures when acid was produced, the actual amount

of acid formed being the difference between the initial and final reactions of the medium.

It is evident that dextrose and lactose are attacked. The small amount of acid formed in the arabinose medium might indicate that there were impurities present which were acted upon while the carbohydrate itself was not attacked. The lot of arabinose used was the best obtainable and had been used for some time with satisfactory results in differentiating the hog-cholera bacillus from the other paratyphoids. If the mouse septicemia bacilli attack it at all they do so very slowly.

Fermented bouillon cultures containing the following carbohydrates have also been titrated and no acid has been found.

Xylose.	Inulin.
Dulcitol.	Salicin.
Maltose.	Dextrin.
Mannitol.	Starch.
Saccharose.	Glycerol.

Reduction.—The one attempt to study the reducing powers of these organisms was a failure as the presence of 1 per cent of either rosalic acid or methyl red apparently inhibited growth.

Pathogenicity.

Considerable work has been done on the virulence of the mouse septicemia bacilli for a variety of animals. Koch (1) found that they produced a fatal disease in white and gray mice, while the field mouse was immune. Loeffler (2) found that pigeons and sparrows were as susceptible as mice, and that frogs, salamanders, chickens, dogs, cats, and white rats were immune. In rabbits an erysipelas-like infection of the ears and a loss in weight followed an intravenous injection of the bacilli, but the animals usually recovered and later were immune. The virulence of the organisms isolated from pigs with rotlauf corresponds to that of the mouse septicemia bacillus except that very freshly isolated cultures may infect swine. This virulence is soon lost and the cultures are then the same as the organism isolated by Koch.

The strains which I have isolated are all virulent for white mice in as small amounts as 0.001 cc. of a 24 hour bouillon culture. The mice

are usually dead by the 3rd day. The culture isolated by Smith 26 years ago is also virulent for mice in the same amounts, but the animals die from 2 to 3 days later. These cultures when injected subcutaneously into pigeons cause death in about 4 days. Intravenous injection into rabbits causes a marked edema of the ear on the side of the injection and at times the opposite ear is also involved. The animals are very quiet, show a rise in temperature to around 41°C., and lose from 150 to 250 gm. in weight. Only one of the cultures will kill rabbits when injected either intravenously or subcutaneously. Whereas in mice and pigeons after death the organisms are abundant in all the organs and in the blood stream, in rabbits they are very scarce. They are rarely found in films, and in cultures made from bits of the various organs, or from several drops of blood, growth in agar slants occurs only in the condensation water.

Three pigs have been inoculated with the cultures isolated from the tonsils. One was given an intravenous injection of 1 cc. of a 24 hour bouillon culture. There was a slight rise in temperature without any signs of illness. The skin was normal and the appetite undiminished. The pig was then inoculated with hog-cholera virus and at autopsy cultures were made from the liver, spleen, and kidney, with negative results.

Two pigs were inoculated with another strain, one receiving 5 cc. of a 24 hour bouillon culture intramuscularly and the other 4 cc. intraperitoneally. Neither pig showed any effects from the inoculation. These results correspond to those obtained by Smith (5) and Moore (4) with the mouse septicemia bacilli which they isolated in this country.

One of the striking features of films made from mice and pigeons is the great number of organisms that is found in leucocytes. Koch (1) first called attention to this, and it has since been used as one of the means of identifying these organisms. Curiously enough, I have found no statement as to the type of cells in which these bacteria are found. From the drawings of Koch (1) and Moore (4) one might assume that they are polymorphonuclear leucocytes, since some of the cells show two nuclei. In the literature they are called leucocytes without further qualification.

Several years ago I made some experiments with an old stock culture of the mouse septicemia bacillus, one of the objects being to determine in which type of cell it occurred. After a subcutaneous injection of about 0.01 cc. of a 24 hour bouillon culture mice died on the 4th or 5th day. A number of inoculated mice were killed at intervals, and films, sections, and cultures made from the various organs. Cultures showed that the organisms were generally distributed at the end of $2\frac{1}{2}$ days, but they were so scarce that they could not be found in films. On the 4th day, when the animals appeared to be sick, the bacilli could be found in films, especially in those from the lungs. Except when present in great numbers, they were always intracellular, and the only type of cell in which they could be found was the endothelial leucocyte (Figs. 1 and 2). Sections of the various organs showed that the bacilli at this stage were in the endothelial cells lining the veins and capillaries and also in endothelial cells free in the blood stream (Figs. 4 and 5). They were not found in endothelial cells lining the arteries or the heart. As the time of death approached the organisms were present in enormous numbers, filling the endothelial cells and crowding the nucleus to one side so that the cell resembled a sac containing a culture of the bacilli. When films were made these sacs were ruptured and the organisms were set free.

In pigeons the process was apparently the same except that the organisms were found generally distributed about 24 hours earlier than in the mouse. They were in the endothelial cells lining the veins and capillaries, and the type of cell in the blood containing them was apparently an endothelial leucocyte (Fig. 3). In both the mouse and the pigeon the organisms were at no time found in polymorphonuclear leucocytes.

Endothelial cells with only a few bacteria soon show evidences of injury. The cytoplasm contains small vacuoles and the nucleus when stained with a modified Romanowsky stain has a more reddish tinge than the nucleus of the same type of cell that is free from bacteria. As the bacilli become more numerous larger vacuoles appear in the cytoplasm and the nucleus stains distinctly red and shows evidences of disintegration.

The disease in the mouse appears, then, to be associated with an intracellular process. The organisms are taken up and instead of

being destroyed are able to multiply and finally to kill the cell. Some of these infected endothelial cells probably break away from the walls of the vessels and float free in the blood stream. Rous and Jones (23) have called attention to the fact that phagocytosed bacteria may be protected against immune substances in the body fluids. There are other instances of the multiplication of bacteria in cells. The leprosy bacillus may be found in the endothelial cells of blood vessels and the tubercle bacillus and *Treponema pallidum* are usually intracellular. Smith (24) has called attention to the localization of *Bacillus abortus* in the chorionic epithelial cells of cattle, and Tyzzer (25) to a disease of Japanese waltzing mice in which the organisms are found in the liver cells and cells of the intestinal mucosa. It is worthy of note that most of the diseases in which the organisms are found in cells are more or less slowly progressive, while the disease in the mouse produced by *Bacillus murisepticus* is acute.

SUMMARY AND CONCLUSIONS.

In the United States, organisms, which culturally are mouse septicemia or swine erysipelas bacilli, have been isolated from the tonsils of five of sixteen pigs examined. These pigs all had hog-cholera, but it is probable that the bacilli were in the tonsils before they were infected with hog-cholera, and there is no evidence that they played any part in the disease. The distribution of the infection seemed to be restricted as most of the pigs from which the bacilli were obtained came from one litter. As we do not have clinical rotlauf, or swine erysipelas, in this country, as these organisms, in Europe, have been found in a large percentage of apparently normal swine, and as the disease is produced with difficulty by the injection of cultures, the question may be raised whether they are not secondary invaders rather than the primary cause of the disease with which they have been associated, or else whether the resistance of swine on the European continent does not differ from that of our breeds as a result of differences in foods.

It is possible that the mouse septicemia bacilli found in this country may differ culturally from those present in animals with swine erysipelas. With this in mind, the carbohydrate reactions, as well as

other cultural characters not necessary for the identification of the bacilli isolated, have been studied.

The disease produced by the injection of these bacilli into mice and pigeons has been studied and shown to be largely an intracellular process. The organisms are taken up by the endothelial cells lining the veins and capillaries; there they multiply and soon kill the cells. It has also been shown that the only type of cell in the blood stream which contains bacteria is the endothelial leucocyte, and the probabilities are that the free phagocytes have been detached from the lining of the vessels. The disease is acute, and the indications are that in the cells the bacilli find a favorable medium for their growth. While phagocytosis may in general be an immune reaction, in this case it appears to favor the parasite rather than the host.

The writer is indebted to Mr. Henry Hagens, of this laboratory, for valuable technical assistance.

BIBLIOGRAPHY.

1. Koch, R., Untersuchungen über die Aetiologie der Wundinfektionskrankheiten, Leipsic, 1878.
2. Loeffler, *Arb. k. Gsndhtsamte*, 1886, i, 46.
3. Smith, T., *U. S. Dept. Agric., Bureau Animal Industry, 2nd Ann. Rep.*, 1886, 196.
4. Moore, V. A., *J. Comp. Med. and Vet. Arch.*, 1892, xiii, 333.
5. Smith, T., *U. S. Dept. Agric., Bureau Animal Industry, 12th and 13th Ann. Rep.*, 1897, 166.
6. Pasteur, *Compt. rend. Acad.*, 1882, xcv, 1120. Pasteur and Thuillier, *Compt. rend. Acad.*, 1883, xcvii, 1163.
7. Schütz, *Arb. k. Gsndhtsamte*, 1886, i, 56.
8. Smith, T., *U. S. Dept. Agric., Bureau Animal Industry, 2nd Ann. Rep.*, 1886, 187.
9. Olt, *Deutsch. tierärztl. Woch.*, 1901, ix, 41.
10. Rosenbach, F. J., *Z. Hyg. u. Infektionskrankh.*, 1909, lxiii, 343.
11. Preisz, H., in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1913, vi, 8.
12. Rickmann, *Z. Hyg. u. Infektionskrankh.*, 1909, lxiv, 362.
13. Stickdorn, W., *Centr. Bakteriol., 1te Abt., Orig.*, 1909, l, 5.
14. Schipp, C., *Deutsch. tierärztl. Woch.*, 1910, xviii, 97.
15. Poels, J., *Folia microbiol.*, 1913, ii, 1.

16. Bauermeister, C., Inaugural dissertation, Ueber das ständige Vorkommen pathogener Mikroorganismen, insbesondere der Rothlaufbacillen, in den Tonsillen des Schweines, Wolgast, 1901.
17. Van Velzen, P. A., Inaugural dissertation, Das Vorkommen pathogener Mikro-Organismen bei gesunden Schweinen, The Hague, 1907.
18. Pitt, W., *Centr. Bakteriol., 1te Abt., Orig.*, 1907, xlv, 33, 111.
19. Loeffler, F., *Mitt. k. Gsndhtsamte*, 1881, i, 134.
20. Petri, R. I., and Maassen, A., *Arb. k. Gsndhtsamte*, 1893, viii, 318.
21. Van Nederveen, H. J., *Folia microbiol.*, 1913, ii, 10.
22. Fermi, C., *Centr. Bakteriol.*, 1892, xii, 713.
23. Rous, P., and Jones, F. S., *J. Exp. Med.*, 1916, xxiii, 601.
24. Smith, T., *J. Exp. Med.*, 1919, xxix, 451.
25. Tyzzer, E. E., *J. Med. Research*, 1917–18, xxxvii, 307.

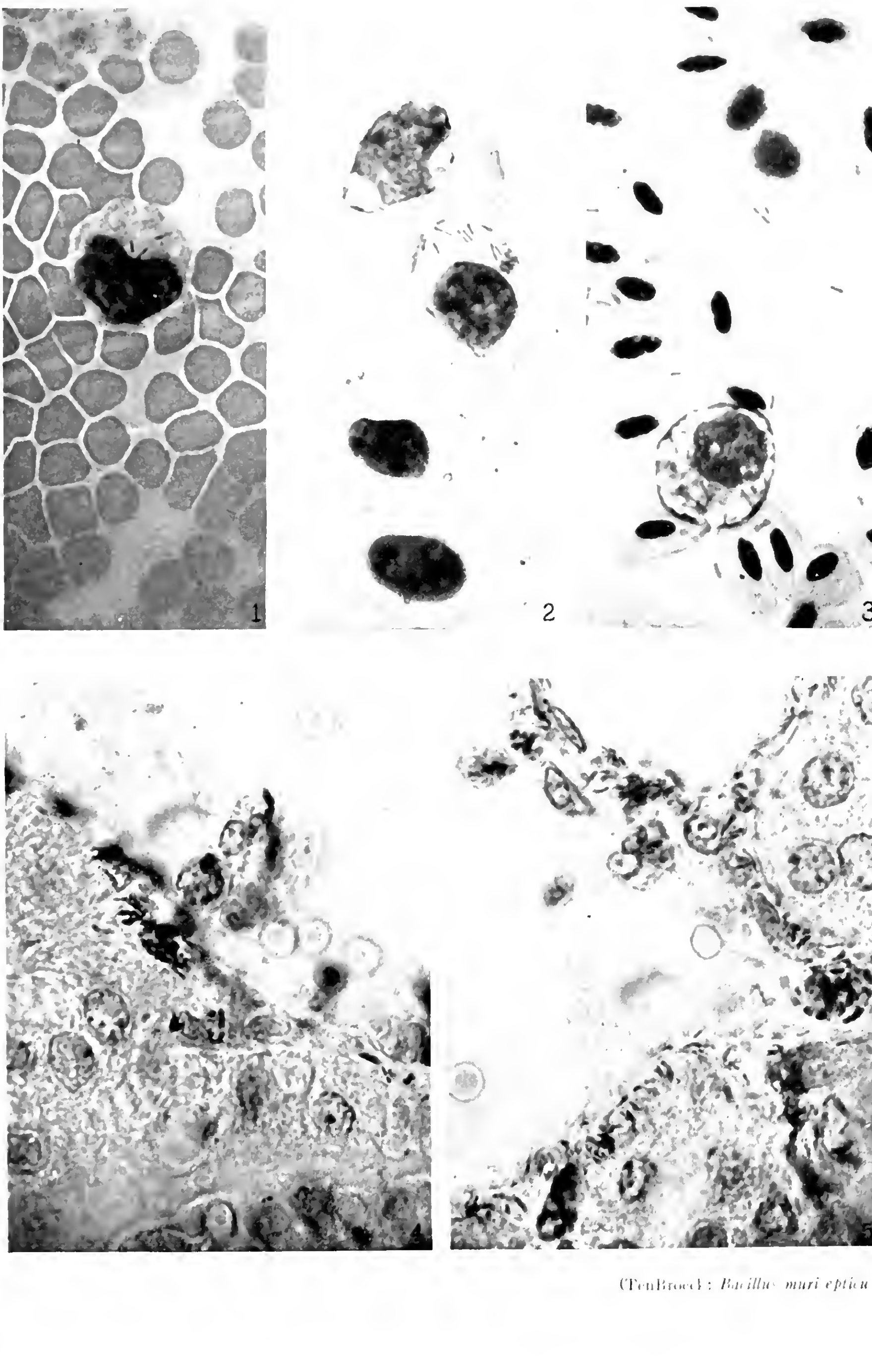
EXPLANATION OF PLATE 24.

Fig. 1. Film from the heart's blood of a mouse killed 3 days after the subcutaneous injection of 0.01 cc. of a bouillon culture of freshly isolated *B. murisepticus*. Bacilli in an endothelial cell. × 1,000.

Fig. 2. Film from the lung of the same mouse. Bacilli in two endothelial cells. × 1,000.

Fig. 3. Film from the lung of a pigeon dead after a subcutaneous injection of 0.01 cc. of a bouillon culture of freshly isolated *B. murisepticus*. Bacilli in a mononuclear cell which is probably an endothelial leucocyte. Free organisms from ruptured cells. × 1,000.

Figs. 4 and 5. Sections of the kidney of a mouse showing *B. murisepticus* in the endothelial cells of a vein. × 1,000.



(TenBroeck: *Bacillus murisepticus*.)

EFFECTS OF ENZYMES IN SERUM ON CARBOHYDRATES AND THEIR RELATION TO BACTERIOLOGICAL TECHNIQUE.

By CARL TENBROECK, M.D.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

(Received for publication, May 21, 1920.)

While studying the carbohydrate reactions of the mouse septicemia bacillus (1) a source of error was detected which apparently has not been appreciated by some bacteriologists and to which it is the object of this paper to call attention. These organisms grow better when a small amount of serum is added to media. Therefore, according to the custom of those who have been working with streptococci, a few drops of sterile horse serum were added to each tube of carbohydrate bouillon at the time the inoculation was made. Under these conditions acid was produced from dextrin and starch, whereas some of the simpler carbohydrates were not attacked. The possibility that this action might be due to enzymes in the serum which made the carbohydrates available to the bacteria was considered and the following experiments were made.

That various sera contain carbohydrate-splitting enzymes has been known to physiological chemists for years and the fact is stated in a number of text-books. Magendie (2) in 1846 found a starch-splitting enzyme in serum and Hamburger (3) showed that in addition to a diastase, a maltase was present. Moeckel and Rost (4) studied the sera of most of the domestic animals and found that they all contained a diastase. Invertase may be present in small amounts in some sera, though Abderhalden and Rathsmann (5) did not find it in dog serum until after they had injected saccharose into the circulation.

Among bacteriologists, Hiss specifically states that serum water should be heated to destroy the enzymes before the carbohydrate is added. Holman (6) found that streptococci grow better when unheated serum is used, and he introduced a medium containing 20 per cent of serum that is sterilized by filtration. Park and Williams (7) add an equal amount of sterile serum or ascitic fluid to a previously sterilized peptone water containing the carbohydrate to be used.

While the use of serum waters for the detection of acid is being gradually replaced by more accurate quantitative methods, the practice of adding a few drops of serum as an enriching substance is not at all uncommon. That this practice may give rise to misinterpretations is indicated by the following experiment.

Experiment 1.—Four fermentation tubes were prepared, one containing sugar-free bouillon, and the other three the same bouillon plus 1 per cent of Kahlbaum's soluble starch. Horse serum was added as indicated in Table I and the tubes were incubated over night to insure sterility. They were then inoculated from a bouillon culture of Morgan's Bacillus No. 1, an organism that attacks only the hexoses. After 5 days incubation the amount of gas was recorded and the amount of titratable acid was determined in the fluid of the bulb and of the branch of each tube. The results obtained are given in Table I and show that in the presence of serum or starch alone gas and acid are not formed, whereas when both of these substances are present both gas and acid are produced.

TABLE I.

Effect of Horse Serum on Starch.

Fermentation tube containing sugar-free bouillon plus.		Results of inoculation with Morgan's Bacillus No. 1 and incubation for 5 days.		
Starch.	Serum.	Gas.	Reaction of.	
			Bulb.	Branch.
per cent		*per cent*		
1	None.	None.	Neutral.	Acid, 1.3 per cent.
1	1 cc. = 4 per cent.	30	Acid, 2.3 per cent.	" 3.5 " "
1	2 " = 8 " "	23	" 3.7 " "	" 3.3 " "
None.	2 " = 8 " "	Bubble.	" 0.5 " "	" 1.3 " "

Since in the above experiment the smallest amount of serum used was approximately 4 per cent, another experiment was made to determine the least amount of serum that would act on starch.

Experiment 2.—To tubes containing 4 cc. of sterile 1 per cent soluble starch was added 1 cc. of various dilutions of sterile horse serum. After 2 days incubation 0.5 cc. from each tube was transferred to a tube containing 5 cc. of Benedict's solution and the tube heated to boiling in a water bath. Tests for bacterial growth in the original tubes were also made. The results of this experiment, given in Table II, show that 1.2 per cent of serum will attack the starch to such a de-

gree that in 2 days there are substances present that will give marked reduction of Benedict's solution, and with as little as 0.3 per cent serum there is still evidence of an hydrolysis of the starch.

That these enzymes are not affected by keeping sera in the refrigerator for a considerable length of time is shown by the fact that the serum of a horse, a cow, and a pig, each $1\frac{1}{2}$ years old, when added to starch solution caused, after 2 days incubation, the production of substances that reduced Benedict's solution. Heating horse serum

TABLE II.

Amount of Serum Required to Produce Reducing Substances for Starch.

Tube.	4 cc. of 1 per cent starch plus 1 cc. of horse serum.			Result of transferring 0.5 cc. to 5 cc. of Benedict's solution and boiling.	Test for bacteria growth.
	Dilution.	Serum.			
		per cent			
A	Undiluted.	20		Marked reduction.	Negative.
B	1:2	10		" "	"
C	1:4	5		" "	"
D	1:8	2.5		Reduction.	"
E	1:16	1.2	Incubated	"	"
F	1:32	0.6	2 days.	Slight reduction.	"
G	1:64	0.3		" "	"
H	1 cc. of distilled water (control).			No reduction.	"
I	1 cc. of serum plus 4 cc. of distilled water (control).			" "	"

to 50°C. for 15 minutes did not destroy the enzymes, but serum heated to 53° for 1 hour or 60° for 15 minutes no longer formed reducing substances in starch solution.

The effect of serum on carbohydrates other than starch was tested bacteriologically as follows:

Experiment 3.—Tubes, each containing 9 cc. of fermented bouillon, were autoclaved, and to sets of four was added 1 cc. of previously autoclaved aqueous 10 per cent solution of a carbohydrate to a tube. Eleven of the more commonly used carbohydrates were available for the study. It was realized that some of them, such as the pentoses and alcohols, would probably not be changed by the serum. To each of two tubes of a set was added 0.5 cc. of sterile horse serum, and all the tubes

were incubated for 3 days. Two tubes from each set, one with and one without serum, were then inoculated from a bouillon culture of Morgan's Bacillus No. 1 and the other tubes from a culture of *B. dysenteriæ* Shiga; both of these attack only the hexoses. After a further incubation of 2 days the contents of each tube were titrated and the results given in Table III were obtained.

These results show that serum will change maltose and dextrin so that they give the same bacteriological reactions as dextrose. There is a slight breaking down of saccharose, but other tests have shown that it is of little importance, and while it is possible that other carbohydrates may be changed though not broken down

TABLE III.

Effect of Horse Serum on Various Carbohydrates.

Fermented bouillon plus 1 per cent.	Titratable acid after inoculation with.			
	Morgan's Bacillus No. 1.		*B. dysenteriæ* Shiga.	
	5 per cent serum.	No serum.	5 per cent serum.	No serum.
	per cent	*per cent*	*per cent*	*per cent*
Arabinose	1.0	0.8	1.1	1.1
Xylose	1.0	1.0	0.9	No growth.
Mannitol	0.7	0.5	1.0	0.9
Dulcitol	0.7	0.6	1.1	1.0
Lactose	0.8	0.8	1.0	0.9
Saccharose	1.7	0.7	1.3	1.0
Maltose	4.4	0.5	3.8	1.0
Raffinose	0.8	0.6	0.9	0.7
Inulin	1.0	0.5	1.3	1.2
Dextrin	3.0	0.8	3.9	0.9
Salicin	0.8	0.6	1.1	1.1
Distilled water	0.8	0.6	0.9	0.8

to a hexose, we have no evidence that this takes place. It is fortunate that inulin and salicin are not attacked, as these two carbohydrates have been used so much in differentiating streptococci.

Maltose, dextrin, and starch are, then, in the presence of unheated serum, of no value in differentiating bacteria. They may, however, be of great value when used without serum. Maltose has not been generally used, since it often gives the same results as dextrose. In separating the fowl typhoid from *Bacillus pullorum*, maltose is of great value and it also helps in classifying the dysentery bacilli.

CONCLUSIONS.

It has been shown that enzymes in serum will change maltose, dextrin, and starch so that they will react as dextrose in media. These enzymes are destroyed by heating to 60°C. for 15 minutes, but they are present in sera that have been refrigerated for as long as 18 months. The practice of using carbohydrate media containing unheated serum should be discouraged, and if it is used the possibility that the carbohydrate may be changed by the enzymes present must be considered.

The writer is indebted to Mr. Henry Hagens, of this laboratory, for valuable technical assistance.

BIBLIOGRAPHY.

1. TenBroeck, C., *J. Exp. Med.*, 1920, xxxii, 331.
2. Magendie, M., *Compt. rend. Acad.*, 1846, xxiii, 189.
3. Hamburger, C., *Arch. ges. Physiol.*, 1895, lx, 543.
4. Moeckel, K., and Rost, F., *Z. physiol. Chem.*, 1910, lxvii, 434.
5. Abderhalden, E., and Rathsmann, E., *Z. physiol. Chem.*, 1911, lxxi, 367.
6. Holman, W. L., *J. Infect. Dis.*, 1914, xv, 209.
7. Park, W. H., and Williams, A. W., Pathogenic microorganisms, New York and Philadelphia, 6th edition, 1917, 103.

A MODIFICATION OF VAN LEERSUM'S BLOODLESS METHOD FOR RECORDING BLOOD PRESSURES IN ANIMALS.

By ALFRED E. COHN, M.D., and ROBERT L. LEVY, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

Plates 25 to 28.

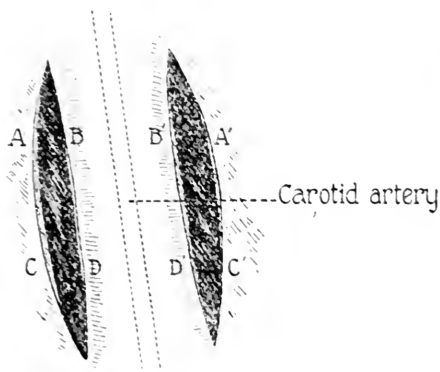
(Received for publication, May 17, 1920.)

A satisfactory method does not exist for taking the blood pressure in laboratory animals so that observations can be repeated on successive days over long periods of time. The advantages of possessing such a method are obvious. A single animal may behave differently on different occasions; it may react differently to different members of the same group of agents. A method such as the one described in this communication affords an opportunity for ascertaining the usual behavior of an animal.

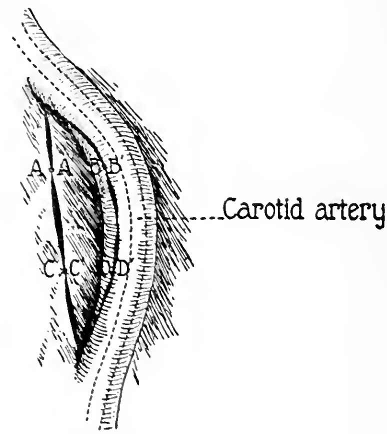
The essential part of the method was described in 1911 by Van Leersum[1] and was utilized by him for studies on rabbits. It consists in making the carotid artery accessible to direct examination. In order to do this an aseptic operation under ether anesthesia is performed. Two longitudinal and parallel incisions are made in the neck of the animals (dogs were utilized by us) about 7 to 10 cm. long (Text-fig. 1), the distance between the two being about 3 to 4 cm. The stretch of skin between the two incisions is freed from the underlying muscle. As much of the subcutaneous tissues is permitted to remain attached to the skin as will insure to it a sufficient blood supply. The carotid artery is then found and gently freed of the other structures contained in the carotid sheath; when possible a generous amount of areolar tissue is permitted to continue to surround the artery. The length of the artery freed in this manner should be

[1] Van Leersum, E. C., Eine Methode zur Erleichterung der Blutdruckmessung bei Tieren, *Arch. ges. Physiol.*, 1911, cxlii, 377.

several centimeters longer than the skin incisions. The edges (Text-fig. 2) of the stretch of skin are next sewed together so that the skin surrounds the artery as a tube. The outer edges of the two incisions are then approximated and sutured to restore the skin of the neck. The adjustment of the edges at the two ends of the tube and of the skin of the neck must be exact. The ligature material may be silk, but we have found retaining sutures of chromic gut and intervening



Text-Fig. 1.



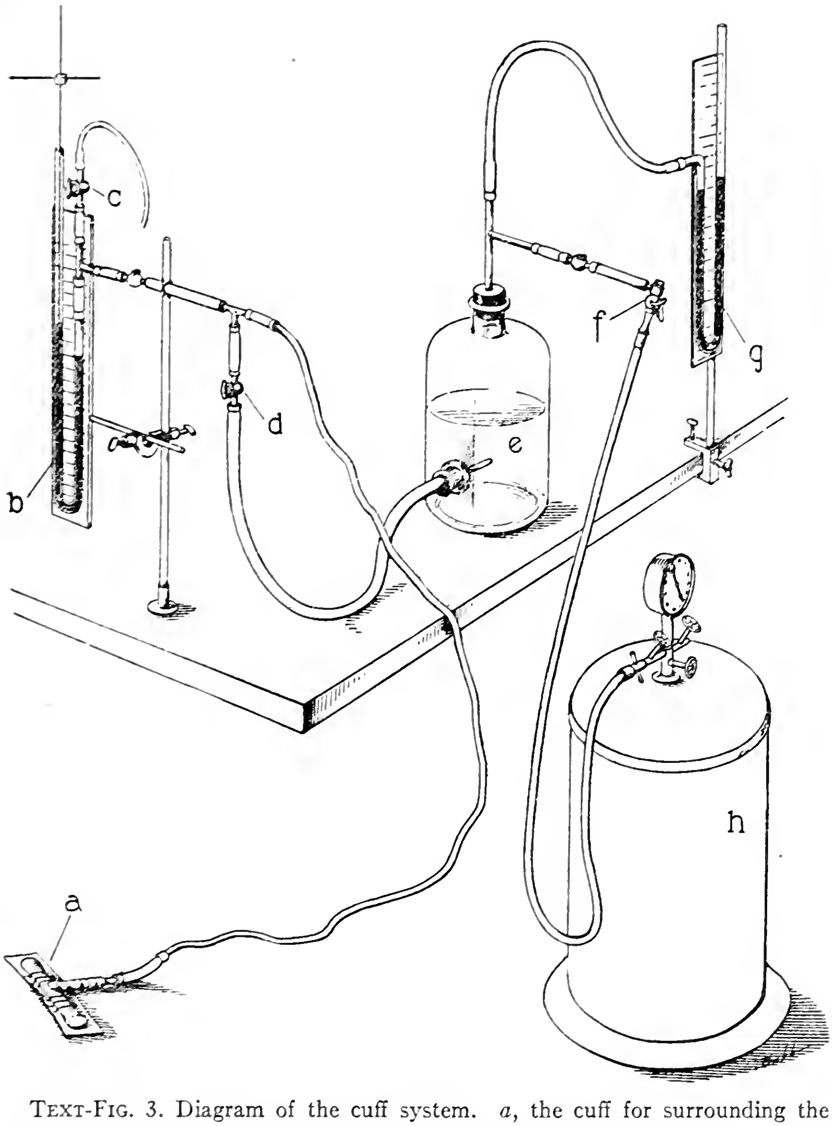
Text-Fig. 2.

Text-Fig. 1. Two vertical and parallel incisions in the neck are shown. The position of the carotid artery in respect to the stretch of skin between them is indicated.

Text-Fig. 2. The formation of the tube of skin containing the carotid artery is shown. After freeing the artery from the carotid sheath it is lifted out and surrounded by the stretch of skin between the incisions shown in Text-fig. 1. The operation is completed by suturing the points *BB′*, *DD′* and *AA′*, *CC′*.

sutures of plain gut more satisfactory. A dry dressing is interposed between the carotid tube and the repaired neck. The wound is dressed with dry gauze and the bandage is held in place with adhesive plaster. The wound may be dressed after an interval of 4 to 6 days. The sutures are removed when sufficient healing has taken place. The wound should heal by primary intention (Fig. 1).

The carotid artery surrounded by its tube of skin is now accessible to examination. In the technique employed by Van Leersum it is



TEXT-FIG. 3. Diagram of the cuff system. *a*, the cuff for surrounding the carotid tube. *b*, mercury manometer and writing point. *c*, valve permitting escape of pressure from the system. *d*, valve for elevating pressure within the system. *e*, water bottle kept at a pressure of about 250 mm. of mercury (*g*). *f*, escape valve. *h*, air pressure tank to supply pressure for *e*.

surrounded by a rubber cuff designed like that of von Recklinghausen but of smaller dimensions, 1 or 2 cm. wide by 3 or 4 cm. long. The pressure in the cuff is elevated until pulsation is no longer felt in the artery distal to the cuff. This pressure is read on a mercury manometer.

We have introduced a graphic method of recording pressure. The system between cuff and manometer is filled with water (Text-fig. 3). Except where it was impossible, we have used lead tubing. Pressure is introduced through a cock (*d*) into the system from a bottle kept at a pressure of about 250 mm. of mercury. It is allowed to escape through a stop-cock (*c*) at the proximal limb of the manometer.

By means of these cocks pressure can be elevated or can be permitted to fall either stepwise (Fig. 2) or gradually (Figs. 3 and 4). The record is made on smoked paper by a writing point supported on a float on the mercury column. The base-line is inscribed. Minimum and maximum oscillations utilized by Erlanger as the points of maximum and minimum pressures may be utilized.[2] We have obtained deflections of sufficient size to permit us to make accurate observations. If it is desirable, it is possible to train dogs to lie quite still for an hour or more without anesthesia or narcotics. To be able to do this has obvious advantages.

We are now making use of the method for the study of drugs of the digitalis series in which we desire to obtain simultaneous blood pressure records and electrocardiograms. The results of these studies will be given in another communication.

[2] To avoid the oscillations due to the shock of the impinging blood stream on the cuff, a second cuff distal to the first may be introduced about the carotid tube. The pressure of this cuff is maintained at the diastolic level. Only when the pressure in the artery is high enough to pass the proximal cuff, will the distal one be affected and a record be inscribed.

EXPLANATION OF PLATES.

Plate 25.

Fig. 1. The carotid tube of one of the dogs after recovery.

Plate 26.

Fig. 2. Below is the base-line. The blood pressure is inscribed by the method of intermittent escape of pressure. The systolic pressure is recorded at the step below the top (202 mm. of mercury); diastolic pressure at 100 mm.

Plate 27.

Fig. 3. Record of blood pressure taken by the method of gradual escape. The systolic pressure is 154 mm. of mercury; the diastolic pressure is 88 mm. The top line is a signal to indicate when an electrocardiogram was made. The bottom is the base-line.

Plate 28.

Fig. 4. Record of blood pressure taken by the method of gradual increase of pressure. The systolic pressure is 160 mm. of mercury; the diastolic pressure is 88 mm. The top line is a signal to indicate when an electrocardiogram was made. The bottom is the base-line.

Fig. 1.

(Cohn and Levy: Blood pressure in animals.)

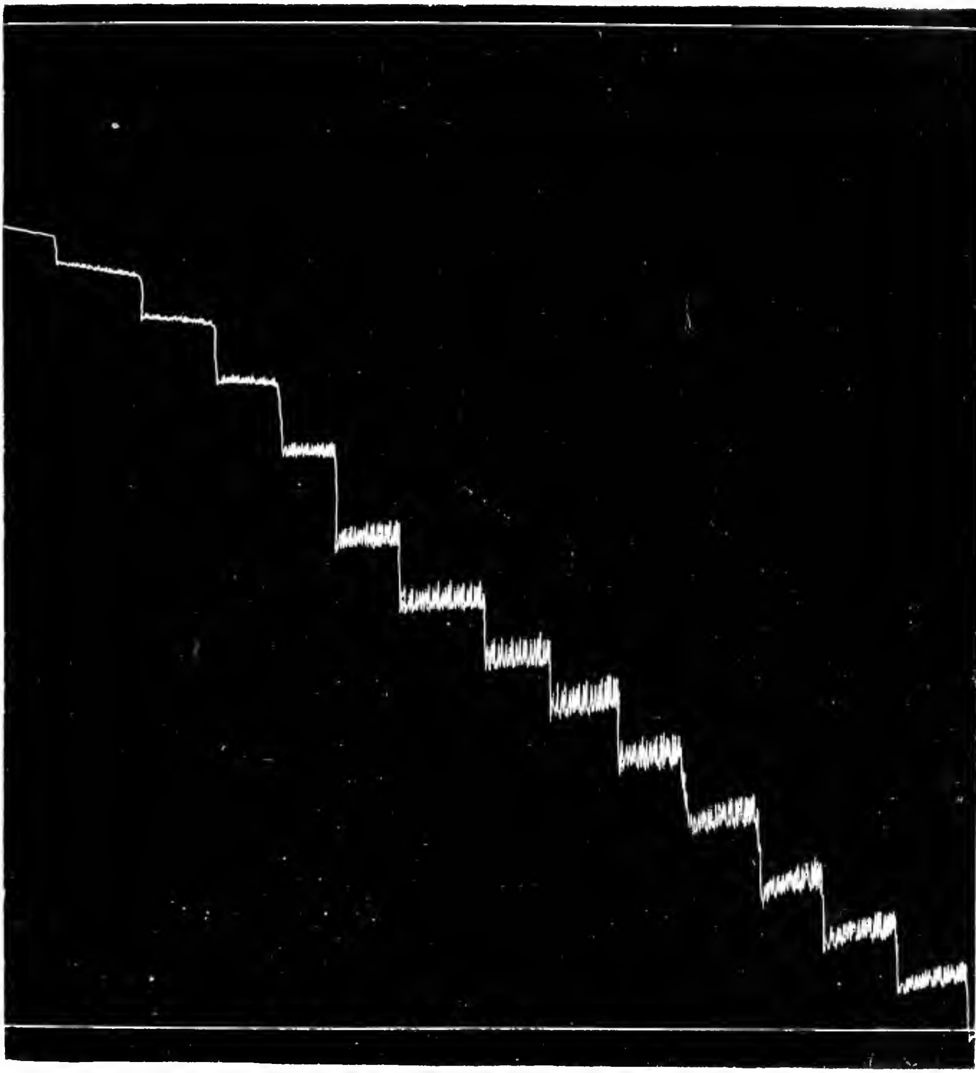


Fig. 2.

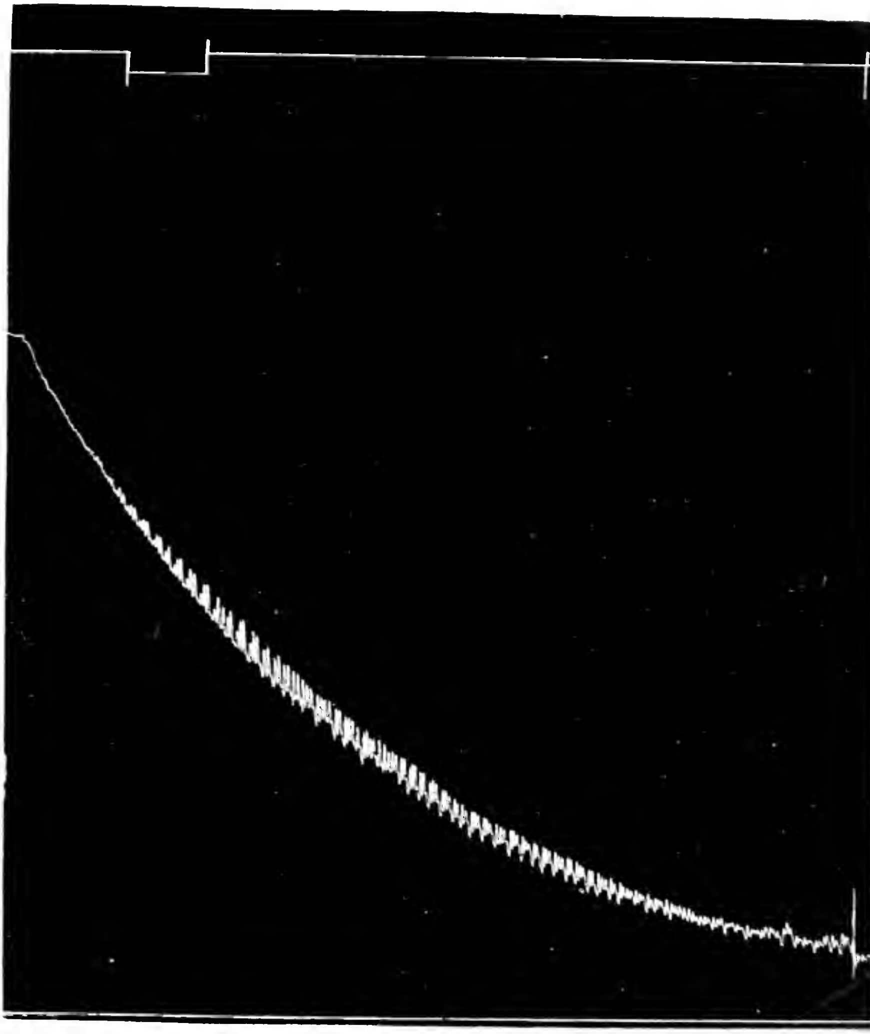


FIG. 3.

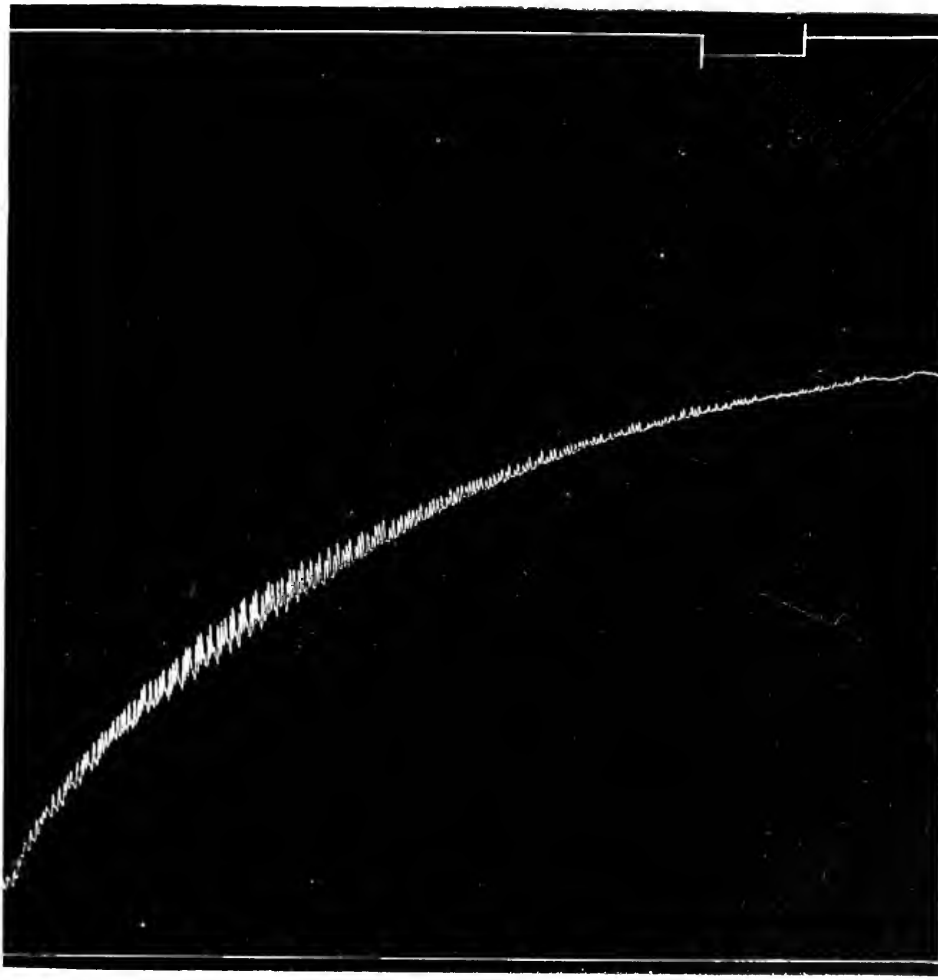


FIG. 4.

(Cohn and Levy: Blood pressure in animals.)

REPRODUCTION IN VITRO OF ENTAMŒBA TETRAGENA AND ENTAMŒBA COLI FROM THEIR CYSTS.

By KAZUYOSHI YOSHIDA, M.D.

(*From the First Medical Clinic of the Imperial University in Kyushu, Fukuoka, Japan.*)

PLATES 29 TO 34.

(Received for publication, December 16, 1919.)

The attempt to cultivate parasitic amebæ on artificial media by employing bacteriological methods, as in the case of non-parasitic amebæ, has produced a voluminous literature during the past few decades. The first cultivation in a fluid medium is claimed by Cunningham (1881). Later Maurice and Walker (1913) attempted to obtain a pure culture of amebæ from a liver abscess and from the intestinal contents of a typhoid patient by the addition of autolyzed liver and kidney to agar plates; but their attempts proved unsuccessful. Williams and Calkins (1913) cultivated amebæ on a rich brain medium; they added daily to this medium a quantity of blood in the presence of hemoglobinophilic bacteria, at a temperature of 38°C. The success of these investigators was due to the fact that they employed living bacteria in the cultivation of amebæ. It is possible that some other types of amebæ have been cultivated from the feces of patients, but whether or not these amebæ were unquestionably parasitic in character is doubtful. Kruse and Pasquale (1894), after numerous trials, concluded that the free living amebæ are capable of cultivation, while the parasitic amebæ are not, but opinion has been divided on this point.

For the past 2 years the writer has endeavored to obtain pure cultures of dysenteric amebæ. Professor Inada suggested that while it is impossible to cultivate successfully the vegetative forms of amebæ because they succumb easily as the bacteria in the medium grow, good results might be achieved with the use of cysts, which are far more resistant than the vegetative forms. On the basis of this suggestion experiments were made at room temperature with cysts relatively free from bacteria. The cysts were placed in a cat liver infusion and cat liver tissue medium according to Carrel's technique, and incubated at 37°C. Unfortunately, because of putrefaction of the culture medium, the experiments were unsuccessful. Since then an attempt has

been made to demonstrate *in vitro* the manner of development within the cyst of the vegetative form. In October, 1917, it was possible to show the reproduction process of the cysts of *Entamœba tetragena* as well as *Entamœba coli.*

On the basis of the experimental results, it may be stated that the cultivation of parasitic amebæ in the human intestinal tract is not at all improbable. The reproduction of these two amebæ takes place by a new process of fertilization; *i.e.*, a kind of autogamy. Moreover, it is evident in the case of these two amebæ that schizogony does not occur, as assumed by Casagrandi and Barbagallo, Schaudinn, and other investigators, for only a single vegetative individual arises from a cyst. Many protozoa pass through a process of sexual fertilization in their developmental cycle, but autogamy has not been observed heretofore with amebæ.

EXPERIMENTAL.

For the successful cultivation of the amebæ from their cysts, it was necessary first to ascertain the resistance of the cysts to certain chemical substances. Kuenen and Schwellengrebel (1913) claim that eosin is a delicate reagent for determining whether or not cysts are alive. Living cysts, they believe, do not stain when placed in a diluted eosin solution, while dead cysts will absorb the eosin instantly. By the aid of this method we were able to prove that bichloride of mercury in a concentration of 1:1,000 kills all the cysts after 4 hours treatment. 50 per cent alcohol and boiling water, and also Schaudinn's sublimate alcohol, kill them instantly. On the other hand, 10 per cent formaldehyde, acting for only a few minutes, does not kill the cysts.

These experiments show, however, that the eosin method does not absolutely establish the death of the cysts. While morphologically the cysts are shown to be dead following the effect of a mercuric chloride, alcohol, or formaldehyde solution, they frequently fail to take the eosin stain. The cysts were kept for 3 minutes in a sublimate solution of 1:1,000 after removal from a tissue or plasma culture medium at room temperature, according to the Carrel method. They were removed from this medium daily and stained with eosin and iron-hematoxylin in accordance with Heidenhain's method. On the 4th or 5th day it was observed that the majority of the cysts did not take

the eosin stain, although their nuclear and plasmic structures in the stained preparations were shown to have degenerated or to have disappeared. From this we may conclude that cysts taking the eosin stain are dead, but not, on the other hand, that all dead cysts absorb this stain.

Uzihara believes that it is possible to determine the resistance of the cysts to chemical agents by a vital stain of diluted neutral red solution, but we were unable to confirm his results. In accordance with Adachi's results, we observed that dead cysts stain slightly with the neutral red, while this is not the case with the living cysts, and that the vegetative forms take only the vital stain. As already stated, we still lack a simple method for determining the resistance of the cysts, and are obliged to obtain this information from animal experiments or the morphological properties revealed by the preparations.

The method followed is described below. A number of cysts were immersed for 5 minutes in a 2 per cent formaldehyde solution, while another set of cysts was immersed for the same length of time in a 2 per cent hydrochloric acid solution. After one washing, the cysts were placed in an isotonic salt solution. The two sets of cysts, together with the control cysts which from the beginning were placed in isotonic salt solution, were kept at room temperature. At intervals of 24 hours a small portion was removed and observed in a preparation stained with eosin or with iron-hematoxylin. In this way it was possible to determine that the majority of the cysts treated with hydrochloric acid, like the control cysts, remained morphologically unchanged; *i.e.*, that the majority of the cysts was not killed by being treated for 5 minutes with a 2 per cent solution of hydrochloric acid.

The material for these investigations was taken from the formed stools of a patient convalescing from amebic dysentery and two carriers of *Entamœba coli.* The feces appeared to be of normal consistency. Numerous cysts were always present in the discharges, but vegetative or other forms of amebæ were absent. It was proved previously that with the use of a special method encystment of the dysenteric amebæ may take place *in vitro* (Yoshida, 1918, *a*). A procedure almost identical with that formerly employed was used also in this case, but the results were negative. With the use of the

same method at a temperature of 28–30°C., we were able to observe *in vitro* the emergence of *coli* amebæ from their cysts; but the results were so meager that we were unable to show the reproductive process in the fixed preparations. Hence it was necessary to find another method, which is described below.

A portion of feces about the size of a thumb joint is placed in a beaker and covered with distilled water, sterile isotonic salt solution, or Ringer's solution, so that it forms a homogeneous mixture of about 40 cc. The water from this mixture is filtered through gauze and the filtrate is distributed in test-tubes and centrifuged by a hand centrifuge.[1] This procedure is repeated several times until the water is quite clear. In order to avoid injuring the cysts in mixing, a pipette with a rubber bulb was employed to admit air and thus distribute the portion of feces evenly.

The cysts are extracted from the sediment by a method based on the difference in specific gravity of the cysts and Mizuame's (Japanese) solution. The procedure is as follows: The sediment is placed in sterile Mizuame's solution, the specific gravity of which is 1,080, then mixed, and centrifuged. As the specific gravity of the cysts of *Entamœba tetragena*, as well as *Entamœba coli*, ranges from 1,060 to 1,070, the cysts rise to the upper layer of water. This layer is removed by means of a sterile pipette, and the cysts are deposited in a sterile test-tube. It is necessary to have sterile apparatus. Distilled water is added until the specific gravity is 1,050. The mixture is then centrifuged. Masses of the cysts will be found in the sediment. These packed cysts are placed for 5 minutes in a 2 per cent hydrochloric acid solution in order to destroy the bacteria adhering to them, without, however, damaging the cysts. The acid is removed by centrifugation. The cysts are then introduced into the water of condensation of the medium described below. The culture is kept at a temperature of 28–30°C. The temperature has a great influence upon the reproduction of the cysts; when it falls below 28°C. reproduction cannot take place. If the temperature is maintained at body heat, the cysts die on the 2nd day. After the cysts have changed into the vegetative forms it is well to keep the temperature under 28°C. in order to inhibit bacterial growth.

[1] An electric centrifuge is not suitable, as its high speed frequently injures the cysts.

A useful culture medium consists of a mixture of two parts of 2 per cent agar and one part of defibrinated horse blood. The agar is placed in an autoclave until it is completely dissolved, after which it is cooled at a temperature of 60°C. While it is still fluid, the defibrinated horse blood is added. The mixture is then slanted and cooled. The success of the experiment depends upon external, as well as internal factors with reference to the cysts; *i.e.*, the resistance of the cysts and the state of the culture medium in regard to putrefaction.

Since November of this year, the cultivation experiment has been repeated thirty times in the manner described above. Three of eleven experiments made on *Entamœba tetragena,* and seven of nineteen on *Entamœba coli* were successful.

After a definite period of time, a small portion of the material was taken from the medium and the amebæ were examined both in their natural state on a warmed stage, and in fixed and stained preparations. Fixation was done in the usual manner in sublimate alcohol; staining was done with iron-hematoxylin, according to Heidenhain and Delafield. Examinations of the living and prepared amebæ were made at first every 5 hours, later twice a day, in the morning and the evening. In the film preparations, it is often difficult to make the material adhere to the cover-glasses, but this difficulty was obviated by placing egg albumin glycerol upon the cover-glasses.

As the morphological changes of the two amebæ resemble each other, they are described together.

Morphological Changes of Entamœba tetragena and Entamœba coli during Reproduction.

Reproduction from these cysts takes place from the 2nd to the 4th day, particularly on the 3rd day. The newly formed amebæ die frequently from the 4th to the 6th day from the effects of putrefaction of the culture medium. Naturally not all the living cysts will reproduce —in fact, the phenomenon proceeds in a relatively small number. Sometimes the cysts die without reproducing at all, while again the process is incomplete. Under favorable conditions the majority of the cysts will reproduce readily *in vitro*. On three occasions out

of nineteen experiments, it was observed that almost all the cysts of *Entamœba coli* changed on the 3rd day into the vegetative form. It is not possible to state conclusively just which cysts will reproduce, but it is probable that only the ripe cysts of both amebæ do so, as it was observed that the unripe cysts die relatively early from the effects of putrefaction of the medium or the hydrochloric acid. Whether or not the newly formed vegetative forms are cultivable remains to be proved.

Two types of reproduction were observed: (1) After a part of the cyst wall has gradually been broken, the young ameba crawls out by means of pseudopodia (Figs. 3 to 6, 8, and 42). In the space left between the cyst membrane and the cytoplasm, light-refracting oval bodies are frequently observed (Fig. 6). It is impossible to say whether these bodies are bacteria or materials extruded from the cytoplasm. The amebæ are observed to stretch out bud-like, homogeneous pseudopodia at one or more parts of the body surface (Fig. 6). It has not been noted that these plasmic extensions become separated; they are always drawn again into the protoplasmic mass. The pictures of *Entamœba coli* presented by Casagrandi and Barbagallo, and also Werner (1908), as illustrating schizogony, should not be interpreted in this manner, as they simply represent the pseudopodia of the amebæ. This mode of reproduction is observed more frequently in *Entamœba coli* than in *Entamœba tetragena*. It may be explained by the fact that the cyst wall of *Entamœba coli* is relatively thicker than that of *Entamœba tetragena*. (2) After the cyst wall has gradually disintegrated and finally disappeared, the ameba flows out by means of its pseudopodia. (*a*) Upon the dissolution of the wall there remains no space between the membrane and the cytoplasm (Figs. 32 and 72). This is the mode of reproduction followed by the majority of both these amebæ. (*b*) A space, small or large, is formed between the outer membrane and the cytoplasm (Figs. 9, 10, 43, and 60). After the disintegration of the wall the ameba emerges. The dimensions of the space thus formed differ greatly, the largest observed being about half the size of the cyst (Figs. 9, 10, and 60).

The young ameba has never been observed to move in its covering, but in fresh preparations slow rolling of the entire cyst against the slight flow in the culture fluid has been noted. It is not clear whether

this is to be ascribed to an independent movement of the cysts or to a bacterial cause.

It is noteworthy that in the case of dead cysts, plasmolysis frequently causes the cytoplasm to shrink from the wall of the cyst, creating a space such as is found in living cysts. It is possible, however, to distinguish readily the dead from the living cysts by morphological characteristics. The living cyst is pale and markedly light-refracting. Its cytoplasm is homogeneous or finely granulated, and it never takes the eosin stain. The dead cyst is more or less light-refracting, its cytoplasm is generally irregular, in large granules, and it appears rigid. The dead cysts which refract light slightly can be stained with eosin while those which are markedly light-refracting do not take the stain.

Not infrequently one encounters a *coli* cyst containing eight nuclei, the cytoplasm of which consists of inner and outer granular layers (Fig. 1). In fixed preparations, the inner layer is seen to consist of a fine network; it stains somewhat more intensely than the outer layer. Often the inner layer has at the periphery a membranous structure which sharply differentiates it from the peculiarly large meshed cytoplasm (Figs. 11 and 12). Upon removal of the cyst membrane, the inner layer, which is markedly light-refracting, escapes (Fig. 13). It is questionable whether or not this structure represents a living individual. This phenomenon, which resembles the endogenous development of *Entamœba tetragena* formerly described, was observed *in vitro* on two occasions.

In spite of numerous attempts, we did not observe the emergence from a single cyst of several daughter amebæ which corresponded to the number of nuclei of the cyst under observation. On the basis of these experiments it may be stated that only a single vegetative individual emerges from the parent cyst. The mature cyst of *Entamœba tetragena* possesses as a rule four nuclei (Fig. 41), and that of *Entamœba coli* eight (Figs. 1 and 2). For this reason, several investigators hold with Schaudinn that four or eight daughter amebæ emerge from the parent cyst, but it became clear from these experiments that the assumptions of these investigators are erroneous.

Occasionally one encounters a *coli* cyst which has its cytoplasm divided by a vacuole into a number of unequal parts (Fig. 14). The

number of these parts does not correspond to the number of nuclei contained within the cyst; some parts may harbor nuclei, while others do not. Hence it is evident that there is no process of schizogony present. Fig. 15 gives the impression, gained chiefly by the shrunken membrane of the dead cyst, that division of the cytoplasm is about to take place. On the other hand, not infrequently one finds dead cysts the granular cytoplasm of which is divided into a number of unequal parts. Probably Darling's illustration of schizogony is based on this phenomenon. Fig. 63 shows a number of ameba-like organisms cultivated *in vitro*. In the body of the cell lies an oval, readily stained portion. These organisms, which like the malaria plasmodia later divide by schizogony into a number of daughter individuals, resemble the so called schizogony forms of *Entamœba coli*, as described by Schaudinn. But the number of daughter amebæ issuing from the mother is always restricted to eight.

The vegetative forms of both amebæ as observed *in vitro* were never smaller than eight microns. With regard to the small amebæ found in feces which have been reported as *Entamœba coli*, we agree with Hartmann. It is questionable whether these forms are *Entamœba coli*. Werner gives the diameter of the smallest forms of *Entamœba coli* as five microns. Darling gives the size of *Entamœba tetragena* as six microns. But as neither Werner nor Craig presents any illustrations of these small forms, we cannot affirm them to be *Entamœba coli*. Casagrandi and Barbagallo give ten microns as the size. Small amebæ of this kind which have been observed frequently are shown in Figs. 35, 61, and 62. It is, however, our opinion that these forms have not arisen through schizogony, but rather by the mode of reproduction described above. We observed previously that the flagellate forms of *Trichomonas* and *Cercomonas* change into the ameboid form in a mixture of serum and Ringer's solution. The ameboid form is always smaller than the smallest form of *Entamœba coli*. In movement and structure, in fact in everything except size and nuclear structure, it shows great resemblance to the vegetative form of the *coli* ameba. Hence it may be assumed that the *coli* ameba is frequently confused with these flagellates.

The nucleus of the living *coli* cyst, because of its chromatin mass, is more readily visible than that of the *tetragena* cyst. In fresh prepa-

rations, the cytoplasm becomes markedly light-refracting and opaque shortly before or after reproduction, which, as in the case of *Amœba albida* of Nägler, renders morphological examination difficult (Figs. 6, 7, and 8). In the cytoplasm granules and vacuoles are also frequently present, so that it is not possible to establish the number of nuclei. But a number of nuclei can be plainly observed in the developed amebæ (Figs. 39 and 40); in most *Entamœba coli* they are closely massed (Fig. 38). In the cytoplasm of the newly formed ameba there are at first few granules, and it appears to be without structure. The differentiation of ectoplasm and endoplasm is indistinct, but later this becomes plain. Rod-like or oval granules, as well as numerous bacteria, are frequently seen in the cytoplasm. Whether these granules represent chromidia or bacteria has not been determined.

The movement of the newly formed amebæ is as a rule not vigorous and proceeds by means of sac-like pseudopodia. No definite direction of movement is seen. It happens not infrequently that in reproduction and under suddenly changed conditions—such as a change of temperature or culture medium—the individual puts out finger-tip pseudopodia (Figs. 6, 52, 53, 55, and 66). As the *coli* ameba is less markedly light-refracting than that of *tetragena*, it is often overlooked. The ectoplasm of *Entamœba coli* has frequently less light-refracting properties than the endoplasm, while these conditions are reversed with respect to *Entamœba tetragena*.

The morphological changes of both amebæ which have been followed in a series of fixed preparations are described below. The nuclei of the cysts extrude large masses of chromatin into the cytoplasm. The chromatin is not divided into chromidial bodies, but lies in fine granules within the cytoplasm. In the beginning the cytoplasm consists of a fine network, later of a coarse, irregular network which stains intensely with hematoxylin (Figs. 21, 25, and 53). At times the preparation takes the hematoxylin and orange stain intensely and is covered with precipitate. The meshes themselves are homogeneous and do not stain. *Entamœba tetragena*, on the whole, does not stain so well as *Entamœba coli*, and its reticular structure is more delicate. The ability to absorb the stain appears to be an individual characteristic; occasionally one sees amebæ which stain quite as well as the small

vegetative forms found in feces (Figs. 17, 45 to 47, 49, 54, and 55), and there are amebæ which stain very intensely with hematoxylin, so that the nucleus is hardly recognizable. In such structures it was possible in two stained preparations to observe the homogeneous pseudopodium (Fig. 36). Sometimes one sees intensely stained amebæ of a dirty color with joint, compact nuclei in the environment of which there is a light zone (Fig. 27). When these amebæ are decolorized, the nuclei are plainly visible. Within the nuclei there is an increasing accumulation of chromatin. The peripheral chromatin zone of the nucleus gradually broadens in the course of the cyclic process, and large masses of chromatin are then extruded into the cytoplasm. The outer chromatin is particularly well developed at this stage, and it is arranged in a half moon or ring-shaped form. The nuclear network is not very distinct. In advanced stages the nuclei are often pycnotically stained because of the marked chromatin accumulation, so that the inner structure is no longer distinguishable.

The multiple nuclei of the cyst then approach one another gradually and merge (autogamy), forming one or two fusion nuclei (syncaryons). The number of nuclei participating in the fusion varies, but as a rule there is a merging of three or four, rarely six nuclei. Four is the usual number in *Entamœba coli*, and from two to four in *Entamœba tetragena*. We observed several *coli* cysts in which two or three nuclei were gathered close together at two points in the cytoplasm (Figs. 32, 33, and 72), and again newly emerged vegetative forms with large nuclei (Figs. 34 and 73). These pictures show that syngamic nuclear union may take place simultaneously at two points within the same individual. This process has never been observed in *Entamœba tetragena*.

As already stated, it has been possible to establish the fact that autogamy takes place in both *Entamœba tetragena* and *Entamœba coli*, not only between two nuclei, but also it may involve more than that number in order to produce a syncaryon, while in the case of other protozoa this process is operative only between two nuclei. In order to distinguish these types of autogamy, the terms simple and polynuclear have been devised. The latter may be further classified as trinuclear, tetranuclear, and pentanuclear autogamy.

Morphological investigation of the structure of the nucleus in reproduction is as a rule very difficult, because of the abundance of

chromatin. But in nuclei relatively poor in chromatin one is able to observe the merging of the nuclear membranes and the formation of a common membrane about the nuclei. The peripheral chromatin masses are then seen to fuse. For this reason at this stage there is seen a syncaryon which still retains the form of the daughter nuclei, so that it is possible to determine the number of daughter nuclei participating in the process of autogamy. Figs. 18, 20, and 68 show four nuclei fusing to form a syncaryon. Figs. 21 to 25, 52 to 56, 66, and 76 illustrate trinuclear autogamy. Figs. 44 to 48 and 75 show the autogamy of two nuclei with sparse chromatin material. It is difficult to follow the changes in the caryosome of the daughter nuclei in reproduction, because of the small amount of chromatin contained in the caryosome, which appears indistinct. It seems probable, however, that the fusion of caryosomes occasionally takes place within the nucleus, for not infrequently one observes what appears to be a fusion of two caryosomes (Figs. 4 and 48). Diminution of the chromatin follows complete union of two or more nuclear parts. The large mass of chromatin of the syncaryon is then dissolved gradually, leaving a residue of peripheral chromatin and the newly formed caryosome. A meshy structure is formed between the outer chromatin and the caryosome.

The syncaryon is at first irregularly formed, and appears flattened, polygonal, almond-shaped, or in the form of a cross, giving the impression of fused nuclei. There is no distinct outline to the nucleus in this stage. With the lessening of the nuclear chromatin, the syncaryon assumes a spherical shape (Figs. 26 and 57), and later it has a structure resembling that of the vegetative forms found in feces (Figs. 31, 58, and 62). The nuclei not participating in the process of autogamy were found to degenerate before or after the syngamic nuclear union; they finally disappear (Figs. 4, 16, 22, 43, 46, and 55) or are extruded from the organism as foreign bodies (Fig. 51).

Similar processes are observed in other protozoa. Von Prowazek has described a sexual cycle in the cyst of *Bodo lacertæ* in which two of the total number of six gametes are autogamically united, while the other four degenerate and disappear. Glaeser noted in regard to the tetranuclear cyst of *Amœba mira* that three nuclei perish, and that the remaining one evidently represents the gamete. Nägler in the

case of the cyst of *Amœba albida* observed a similar process, which consisted in the union of two gametes and the disappearance of the other two nuclei.

The best known case of autogamy is the conjugation of *Actinosphærium eichhorni* so well described by Hertwig. In this polynuclear heliozoan, reproduction is preceded by destruction of a part of the cell nucleus. Within the mother cyst the number of nuclei, from 20 to 500, is reduced to about 5 per cent of the total number. The disappearance of the nuclei is effected by processes of dissolution and resorption. During the past year we have not found any vacuoles in the tetranuclear cysts of *Trichomonas intestinalis* and we were able to determine in the cultivation of these cysts the development of a young ameboid form, the fusion of a number of nuclei, and the degeneration of others, as in *Entamœba tetragena.*

The ejection of the nuclei is a phenomenon which had already been observed by a number of investigators. Wenyon established the extrusion of superfluous nuclear substances with regard to *Entamœba muris.* Von Prowazek demonstrated a number of times the expulsion of nuclei in the vegetative form of *Entamœba williamsi,* and gave an illustration. He described this stage as a particular form of rejuvenation, asexual in character, which is not rare among protozoa. In this procedure, in our opinion, there is solely an expulsion of useless substances. Fig. 16 shows a *coli* cyst which has two degenerated and four closely joined nuclei. Fig. 30 illustrates a vegetative form possessing a large syncaryon and two residual nuclei. It is not difficult to interpret this picture: a number of the closely joined nuclei unite, while the other nuclei, which do not participate, shrink and degenerate. This picture is sometimes encountered in material obtained from the feces of cats infected with the cysts.

These processes of autogamy take place at the reproductive stage or shortly after; *i.e.*, in the newly emerged vegetative forms, as in the case of *Amœba mira,* according to Glaeser. In a number of vegetative forms the nuclei do not conjugate completely. Sometimes the syngamic nuclear union is already present in the cyst, and again one finds cysts possessing developed syncaryons. On the whole it may be said that the nuclei approach one another in the cystic stage, and they unite after reproduction takes place. Hence shortly after reproduc-

tion, the nuclei present the picture of the middle stage of autogamy. Fig. 5 shows a *coli* ameba, which still has six nuclei, emerging from its burst shell. Fig. 43 illustrates an *Entamœba tetragena*, of which two nuclei remain quite isolated, although a part of the membrane has already loosened. Fig. 60 shows a *tetragena* cyst with two nuclei in process of conjugation. Fig. 42 illustrates the emerging of the *tetragena* ameba from the capsule after the completion of the syngamic nuclear union. Fig. 57 shows a newly created *tetragena* ameba, produced by autogamy. Fig. 50 illustrates a newly created vegetative form with four nuclei lying close together, without, however, fertilization. As the syngamic nuclear union is a slow process, it is possible to obtain in stained preparations numerous pictures illustrating autogamy.

The newly created ameba frequently attains the size of the cyst, but again it may be only half as large. Hence, it is obvious that the vegetative form of the *coli* cyst shortly after reproduction is larger than that of *Entamœba tetragena*, inasmuch as the *coli* cyst as a rule is larger than that of *tetragena*. These small amebæ shortly after reproduction take in various bacteria for food (Figs. 22, 44, and 49); and on the 4th day of development they attain the size of the normal vegetative forms found in feces. As during this stage bacterial proliferation is marked, the culture medium undergoes decomposition and becomes chocolate-colored. The amebæ then develop numerous vacuoles, and their nuclei shrink and degenerate (Fig. 59), until they finally burst. On the 5th day of the experiment one rarely encounters large amebæ and the small forms have wholly disappeared.

On the basis of the results described above the conclusion was drawn that the daughter nuclei in the cyst stage correspond exactly to the gamete nuclei of other protozoa. The difference between these gametes and those of other protozoa consists only in the fact that these gametes show no differentiation between male and female, and are enclosed in a common cytoplasm. We have here an example of primitive sexual fertilization. Schaudinn and other investigators have established the fact that the change into a chromidial cell is a normal process in the formation of gametes in a number of protozoa. This may also be assumed with regard to *Entamœba coli* and *Entamœba tetragena*. The fact that the nucleus of the vegetative forms extrudes large masses of chromatin during the formation of the cyst,

and that this chromatin is then developed into small daughter nuclei (gametes), agrees entirely with the views of Schaudinn. We have previously described how in the cyst formation of the atypical forms of *Entamœba tetragena*, the chromatin, or chromidia, extruded from the nucleus into the cytoplasm, later form from one to four daughter nuclei, as has been proved by Popoff to be the case in *Amœba minuta*. The process is in accord with the phenomenon observed in Rhizopoda in which the nuclei of the gametes are reconstructed from chromidia. These facts afford proof that the daughter nuclei of the cysts correspond to the gamete nuclei. Schaudinn has assumed that all protozoan cells are binuclear. He says:

> While in the case of the Infusoria, in Polystomella, Centrophyxis, and Chlamydophyrs, the sex substance of the nucleus during the vegetative period develops separately from the metabolism nucleus, and arises out of a special nucleus or chromidial mass, in the case of *Entamœba coli* it is united with the vegetative nuclear substance in one nucleus. A separation takes place only just before conjugation, when the substance of the metabolism nucleus, or the nucleus itself, is extruded or otherwise destroyed. A chromidium arises and exists for a short period. Out of this the sex nuclei are differentiated.

Nägler also observed in the case of the cyst of *Amœba albida* that a separation of the generative and vegetative nuclei precedes autogamy. According to our observations, chromidial formation in the case of *Entamœba coli* and *Entamœba tetragena* is found more frequently in the gamete stage than in fertilization, and with the exception of a few cases it is not possible to demonstrate unequivocally a reductional division before fertilization takes place. One observes, however, that the nuclei which do not participate in autogamy become disintegrated and that the nuclear chromatin passes into the cytoplasm. In the case of *Entamœba tetragena*, which is related to *Entamœba coli*, the chromidia and the chromatin play a large part in the formation of the gametes, and also on the other hand in the vegetative function. On the basis of these observations we are unable to confirm Schaudinn's theory of nuclear dualism with respect to the protozoan cell. It appears to be logical to assume that there are two kinds of chromatin, as already asserted by Doflein; *i.e.*, idiochromatin and somatochromatin.

On two occasions the author encountered in the culture medium a *coli* cyst having more than eight nuclei (Fig. 37). These nuclei are arranged in pairs, and the pictures show that following the disappearance of a number of nuclei, those remaining rearrange themselves in groups of two. It has not been determined whether or not this represents a reduction process. Hartmann and other investigators have recorded *coli* cysts with more than eight nuclei.

Schaudinn has described a complex nuclear change which occurs in the formation of the cyst of *Entamœba coli.* He claims that syngamic nuclear union occurs only in the tetranuclear stage and is the result of conjugation and the metagamic reproductive processes of a cyst with eight nuclei. In support of Schaudinn's view of autogamy, we have the claims of Wenyon with respect to *Entamœba muris*, which is now assumed to be identical with *Entamœba coli.* Hartmann and Withmore on the basis of their own experience did not admit the claims of Schaudinn and Wenyon, but came to the conclusion that no autogamic process of fertilization within the cyst had been observed. We concur in this view of Hartmann. Wenyon claimed to have made consecutive observations upon living cysts and gave as proof a number of consecutive illustrations of the same ameba. But he described autogamy very briefly, as did Schaudinn, and presented no illustrations of importance. For instance, he did not illustrate the conjugation of the four pairs of cells. Concerning autogamy he wrote:

"The two nuclei of each pair then apparently fused, producing again a cyst with two nuclei. These two nuclei then began to increase in size and almost immediately divided to form four nuclei."

It is clear that no unequivocal proof exists that autogamy has been observed by Wenyon in a living individual. Again he wrote:

"The duration of the spindle formation and conjugation was at most only ten minutes, and this explains the difficulty of finding these stages in fixed preparations."

In the case of other amebæ no such rapid phenomenon of isogamy, anisogamy, or autogamy is known at the present time. He appears to describe an unusual case.

Nägler states that it is impossible to follow the syngamic nuclear union in living specimens of *Amœba albida*, as the process requires

at least 24 hours for its completion. This is also true of *Entamœba coli*. In the cases observed by us, the phenomenon of autogamy required quite a long period of time, and it was possible to follow easily the various stages in the fixed preparations. If the theories of Schaudinn and Wenyon were correct, there would be two formations of gametes in one cyst, which is inadmissible. For that reason their views seem unsound.

A similar statement may be made concerning *Entamœba tetragena*. We believed, like Schaudinn, that, preceding the formation of four daughter nuclei in the cyst, processes similar to those of *Entamœba coli* take place with respect to *Entamœba tetragena*, because of the relation existing between the two amebæ; but we were unable to establish these processes in the encystment of dysentery amebæ *in vitro*. Hartmann claimed at an earlier date to have discovered the process of autogamy in the *tetragena* cyst, but a subsequent study of the subject convinced him that he was not concerned with examples of autogamy, but with a degenerated form. Since that time no one has reported the process with regard to *Entamœba tetragena*. It must be stated, however, that the phenomenon of autogamy in *Entamœba coli* as well as *Entamœba tetragena* is not seen in the early cystic stage, but during reproduction.

To assume the phenomenon of schizogony for *Entamœba coli* also seems erroneous. As already stated, the illustrations of Casagrandi and Barbagallo, as well as those of Werner, do not illustrate schizogony, but the emergence of a *coli* ameba from its cystic membrane. The schematic illustration of Wenyon is based upon conjecture. The illustration of a *tetragena* cyst given by Darling was probably that of a dead cyst, in parts of which the granular cytoplasm had been accidentally separated.

The possibility of heterogamy in the development of these two amebæ will be considered briefly. Previously we were able to demonstrate *in vitro*, in the case of the large chromidial forms of *Entamœba tetragena*, the fusion of two amebæ, as Hartmann and Werner claim to have observed in the case of *Entamœba histolytica*. But this fusion has been encountered very rarely, and a union of the nuclei has not been observed. Hence it is not proper in that case to speak of conjugation, but one should rather regard it as plasmogony. More-

over, we have frequently observed three specimens of the small vegetative forms of the dysenteric amebæ hanging together by thin protoplasmic stems. Such pictures do not represent conjugation, but rapid cell division. Therefore, it was not possible for us to demonstrate heterogamy *in vitro* in the period of time required by the large vegetative form for its changes into the cyst. If heterogamy were to take place in amebæ, we would have to look for it during the period in which the cyst changes into the large vegetative form. Assuming, according to Schaudinn, that a number of daughter amebæ emerge from a single cyst by schizogony, one must furthermore consider that they conjugate in pairs to form a copula, as described by Mercier with regard to *Entamœba blattæ* and by Popoff for *Amœba minuta*. But up to the present time we have not observed this form of schizogony, *in vitro*, or such pictures of conjugation. Von Prowazek with respect to *Entamœba williamsi*, which is related to *Entamœba coli*, indicated heterogamy. He stated:

> A number of times we observed very small amebæ lying close together. In the preparations binuclear stages were seen, in which the nuclei were distinct, and as there were also indications of a reduction process, the assumption of heterogamy seems warranted for this entameba.

These observations of von Prowazek are open to question, and his illustrations which might be interpreted as showing a process of fertilization and division by reduction, correspond to Figs. 30 and 35. They do not illustrate heterogamy, but rather autogamy. The origin of these small nuclei is to be attributed to the fact that the daughter nuclei which might participate in the autogamy finally shrink. Similar pictures are found frequently in the nuclear division of the atypical vegetative forms of *Entamœba tetragena*, shortly before encystment.

Development in Vitro of Entamœba tetragena.

In 1918 (*a*), the author reported in his first communication on the subject that the large vegetative form of dysenteric ameba always passes into a small vegetative form, and produces at times a tetranuclear cyst *in vitro*, and that more or less proliferation takes place when feces showing blood-tinged mucus are kept *in vitro*. In this second communication the writer shows that it is possible, by follow-

ing a definite method, to grow *in vitro* the large vegetative forms from the cysts. The two papers, in which different culture media are described, together present the entire developmental cycle of *Entamœba tetragena in vitro*.

CONCLUSIONS.

1. The vegetative forms of amebæ may be developed *in vitro* from the cysts.

2. The cysts of *Entamœba tetragena* and *Entamœba coli* each produce only a single vegetative individual. It has not been observed *in vitro* that a number of daughter amebæ, corresponding to the number of nuclei present in the cyst, emerge from the mother cyst, as claimed by Casagrandi and Barbagallo, Schaudinn, and a number of other investigators.

3. The daughter nuclei in the cystic stage correspond to the gamete nuclei of other protozoa. The syngamic nuclear union in the case of these two amebæ does not take place in the tetranuclear stage of the cyst, as claimed by Schaudinn and Wenyon, but during reproduction.

4. The process of autogamy of both amebæ is not restricted to two nuclei; it may involve more than two, which unite to form a syncaryon. In the case of all other protozoa, autogamy is restricted to two nuclei.

5. In order to distinguish the two types of autogamy, one has been termed simple autogamy and the other polynuclear autogamy. The latter may again be classified according to the number of nuclei involved, as trinuclear and tetranuclear autogamy.

6. The majority of cysts treated for 5 minutes in a 2 per cent solution of hydrochloric acid survives.

7. It was possible to demonstrate *in vitro* the developmental cycle of *Entamœba tetragena*.

8. No evidence of heterogamy has been observed *in vitro* in *Entamœba tetragena* or *Entamœba coli*.

9. The vegetative form of *Entamœba tetragena*, at a certain stage of its life cycle *in vitro*, shortly after its formation, has not only one or two, but may have as many as three or four nuclei.

It gives me pleasure to acknowledge my indebtedness to Professor K. Miyajima for his study of the preparations here described, and to Professor R. Inada, the Director of the Clinic, and Professor Y. Ido for the constant stimulus and guidance which they have given me in this work.

BIBLIOGRAPHY.

Akashi, M., I. Studien über die Morphologie und Entwicklung der *Entamœba coli* Lösch emendata Schaudinn in Japan. II. Studien über die Ruhramöben in Japan und Nordchina, *Arch. Schiffs- u. Tropen-Hyg.*, 1913, xvii, suppl., 457.

Casagrandi, O., and Barbagallo, P., Ueber die Kultur von Amöben, *Centr. Bakteriol., 1te Abt.*, 1897, xxi, 579. Ricerche biologiche cliniche sult' *Amœba coli* (Lösch), *Bull. Accad. Gioenia, sc. nat. Catania*, 1895, No. 41, 7; *Entamœba hominis s. Amœba coli* (Lösch), *Ann. ig. sper.*, 1897, i, 103.

Couret, M., and Walker, J., The cultivation of amœbæ in pure culture upon autolyzed tissues, *J. Exp. Med.*, 1913, xviii, 252.

Craig, C. F., Parasitic amœbæ of man, Philadelphia and London, 1911.

Cunningham, D. D., On the development of certain microscopic organisms occurring in the intestinal canal, *Quart. J. Micr. Sc.*, 1881, xxi, 234.

Darling, S. T., Budding and other forms in trophozoites of *Entamœba tetragena*, *Arch. Int. Med.*, 1913, xi, 495; Observations on the cysts of *Entamœba tetragena*, 1913, xi, 1; The rectal inoculation of kittens as an aid in determining the identity of pathogenic entamœbæ, *South. Med. J.*, 1913, vi, 509.

Doflein, F., Lehrbuch der Protozoënkunde; eine Darstellung der Naturgeschichte der Protozoen mit besonderer Berücksichtigung der parasitischen und pathogenen Formen, Berlin, 3rd edition, 1911.

Elmassian, M., Sur une nouvelle espèce amibienne chez l'homme, *Entamœba minuta n. sp.*, 1re Mémoire, *Centr. Bakteriol., 1te Abt., Orig.*, 1909, lii, 335.

Hartmann, M., Eine neue Dysenterieamöbe, *Entamœba tetragena* (Viereck) syn. *Entamœba africana* (Hartmann), *Arch. Schiffs- u. Tropen-Hyg.*, 1908, xii, suppl., 217; Untersuchungen über parasitische Amöben. I. *Entamœba histolytica* Schaudinn, *Arch. Protistenk.*, 1910, xviii, 207; II. *Entamœba tetragena* Viereck, 1912, xxiv, 163.

Hartmann, M., and Withmore, E., Untersuchungen über parasitische Amöben. III. *Entamœba coli* Lösch em. Schaudinn, *Arch. Protistenk.*, 1912, xxiv, 182.

Juergens, Zur Kenntnis der Darmamöben und der Amöbenenteritis, *Veröffentl. Geb. Mil.-San. Wes.*, 1902, No. 20, 110.

Kartulis, Ueber Riesen-Amöben (?) bei chronischer Darmentzündung der Aegypter, *Virchows Arch. path. Anat.*, 1885, xcix, 145; Dysenterie (Ruhr), Nothnagel, H., Specielle Pathologie und Therapie, Vienna, 1896, v, pt. 3, 1.

Koidzumi, M., On a new parasitic amœba, *Entamœba nipponica*, found in the intestine of Japanese, *Centr. Bakteriol., 1te Abt., Orig.*, 1909, li, 650.

Kruse, W., and Pasquale, A., Untersuchungen über Dysenterie und Leberabscess, *Z. Hyg. u. Infektionskrankh.*, 1894, xvi, 1.

Kuenen, W. A., and Swellengrebel, N. H., Die Entamöben des Menschen und ihre praktische Bedeutung, *Centr. Bakteriol., 1te Abt., Orig.*, 1913, lxxi, 378; Die pathologische Anatomie der Amöbiasis verglichen mit anderen Formen von Dysenterie, *Arch. Schiffs- u. Tropen-Hyg.*, 1909, xiii, suppl., 437.

Mercier, L., Le cycle evolutif d'*Amœba blattæ* Bütschli, *Arch. Protistenk.*, 1909, xvi, 164.

Nägler, K., Entwicklungsgeschichtliche Studien über Amöben, *Arch. Protistenk.*, 1909, xv, 1.

Nöller, W., *Entamœba aulastomi nov. spec.*, eine neue parasitische Amöbe aus dem Pferdeegel, (Aulastomum gulo Moq.-Tand.), *Arch. Protistenk.*, 1912, xxiv, 195.

von Prowazek, S., Beitrag zur *Entamœba*-Frage, *Arch. Protistenk.*, 1911, xxii, 345; Weiterer Beitrag zur Kenntnis der Entamöben, 1912, xxvi, 241.

Ruge, R., Amœbenruhr, in Mense, C., Handbuch der Tropenkrankheiten, Leipsic, 1906, iii, 1.

Schaudinn, F., Untersuchungen über die Fortpflanzung einiger Rhizopoden, *Arb. k. Gsndhtsamte*, 1903, xix, 547.

Viereck, H., Studien über die in den Tropen erworbene Dysenterie, *Arch. Schiffs- u. Tropen-Hyg.*, 1907, xi, suppl., 1.

Wenyon, C. M., Observations on the protozoa in the intestine of mice, *Arch. Protistenk.*, 1907, i, suppl., 169.

Werner, H., Studien über pathogene Amöben, *Arch. Schiffs- u. Tropen-Hyg.*, 1908, xii, suppl., 419.

Whitmore, E. R., Vorläufige Bemerkungen über Amöben aus Manila und Saigon, *Centr. Bakteriol., 1te Abt., Orig.*, 1911, lviii, 234; Parasitäre und freilebende Amöben aus Manila und Saigon und ihre Beziehungen zur Dysenterie, *Arch. Protistenk.*, 1911, xxiii, 71.

Wülker, G., Die Technik der Amöbenzüchtung, *Centr. Bakteriol., 1te Abt., Ref.*, 1911, l, 577.

Yoshida, K., The encystment of dysentery amebæ *in vitro*, *J. Exp. Med.*, 1918, *a*, xxviii, 387; Ueber die Dauercyste von *Trichomonas intestinalis* und ihren Entwicklungskreis *in vitro*, *Nippon Naika Gakkai Zasshi*, 1918, *b*, vi, No. 9.

EXPLANATION OF PLATES.

PLATE 29.

FIGS. 1 to 20. *Entamœba coli.*

FIG. 1. Cyst with eight nuclei. The cytoplasm consists of outer and inner granular layers.

FIG. 2. Ripe cyst containing eight nuclei.

FIGS. 3 to 5. Reproduction of cysts. (Stained preparations.)

FIGS. 6 to 8. Reproduction of cysts. (Fresh preparations.)

FIGS. 9 and 10. Cysts having a small zone between the cyst membrane and the cytoplasm. (Stained preparations.)

FIG. 11. The inner layer of the cytoplasm shows at the periphery a membranous structure, which serves to differentiate the coarse meshed cytoplasm from the inner layer. (Fresh preparation.)

FIG. 12. The same. (Stained preparation.)

FIG. 13. Rupture of cyst membrane shown in Figs. 11 and 12.

FIG. 14. Cyst showing eight nuclei. The cytoplasm is divided by a vacuole into a number of unequal parts.

FIG. 15. Dead cyst. The wall of the cyst shows folds caused by shrinkage. (Fresh preparation.)

FIG. 16. Cyst shortly before reproduction. Four nuclei are intact; three nuclei are degenerated.

FIGS. 17 to 20. Tetranuclear autogamy. Fig. 20 is a vegetative form with syncaryon.

PLATE 30.

FIGS. 21 to 40. *Entamœba coli.*

FIGS. 21 to 25. Vegetative forms, showing trinuclear autogamy.

FIG. 26. Vegetative form with syncaryon.

FIGS. 27 to 29. Vegetative forms showing autogamy involving more than four nuclei.

FIG. 30. Vegetative form with syncaryon and two degenerated gamete nuclei.

FIG. 31. Newly created vegetative form.

FIGS. 32 and 33. Vegetative forms showing autogamy at two points. Fig. 32 is a vegetative form with residual cyst membrane.

FIGS. 34 and 35. Vegetative forms with two syncaryons.

FIG. 36. Vegetative form deeply stained with hematoxylin.

FIG. 37. Cyst having more than eight nuclei.

FIGS. 38 to 40. Reproduced vegetative forms. (Fresh preparations.)

PLATE 31.

FIGS. 41 to 62. *Entamœba tetragena.*

FIG. 41. Tetranuclear cyst of *Entamœba tetragena.*

FIG. 42. Reproduction of *tetragena* cyst, upper part showing cyst membrane, lower portion the vegetative form.

FIG. 43. Cyst shortly before reproduction. Two nuclei are intact, while two others are degenerated.

FIGS. 44 to 49. Binuclear autogamy. Fig. 44 is a vegetative form with three vacuoles, containing bacteria. Fig. 49 is a vegetative form with syncaryon produced by binuclear autogamy.

FIG. 50. Vegetative form with four nuclei.

FIG. 51. Vegetative form, showing extrusion of a gamete nucleus.

FIGS. 52 to 56. Vegetative forms, showing trinuclear autogamy. Fig. 56 is a vegetative form which has completed trinuclear autogamy.

FIGS. 57 and 58. Vegetative forms with syncaryons.

FIG. 59. Degenerated form observed *in vitro* on 4th day of experiment.

FIG. 60. Cyst in autogamy.

FIGS. 61 and 62. Small vegetative forms.

FIG. 63, *a*, *b*, and *c*. Ameboid organisms.

PLATE 32.

FIGS. 64 to 71. *Entamœba coli.*

FIGS. 64 and 65. Reproduction of *Entamœba coli.* × 900.

FIG. 66. Vegetative form, showing trinuclear autogamy. × 900.

FIG. 67. Vegetative form, showing tetranuclear autogamy. × 900.

FIG. 68. Vegetative form with syncaryon produced by tetranuclear autogamy. × 900.

FIG. 69. Vegetative form, showing autogamy of more than four nuclei. × 900.

FIG. 70. Same as Fig. 30. × 900.

FIG. 71. Vegetative form with a syncaryon. × 900.

PLATE 33.

FIGS. 72 and 73. *Entamœba coli.* × 900.

FIG. 72. Same as Fig. 32.

FIG. 73. Vegetative form with two syncaryons.

FIGS. 74 to 77. *Entamœba tetragena.* × 900.

FIG. 74. Reproduction of cyst, upper portion showing membrane, lower portion a reproduced individual. × 900.

FIG. 75. Vegetative form, showing binuclear autogamy. × 900.

FIG. 76. Vegetative form, showing trinuclear autogamy.

FIG. 77. Two new vegetative forms.

PLATE 34.

Development of *Entamœba tetragena in vitro.*

1 to 17. Typical forms.

18 to 27. Atypical forms.

1. Large vegetative form.
2. Transitional form.
3. Small vegetative form.
4. Small vegetative form (chromidial form).

5 to 10. Cysts.

5. Mononuclear cyst with central vacuole.
6. Mononuclear cyst. Mitosis.
7. Binuclear cyst.

8. Trinuclear cyst. Mitosis.

9. Tetranuclear cyst.

10. Tetranuclear cyst. Two nuclei are intact, while two others are degenerating.

11. Reproduction of cyst. Binuclear autogamy.

12. Reproduced vegetative form. Binuclear autogamy.

13 to 15. Trinuclear autogamy.

15. Reproduced vegetative form with syncaryon and one shrunken nucleus.

16 and 17. Young vegetative forms.

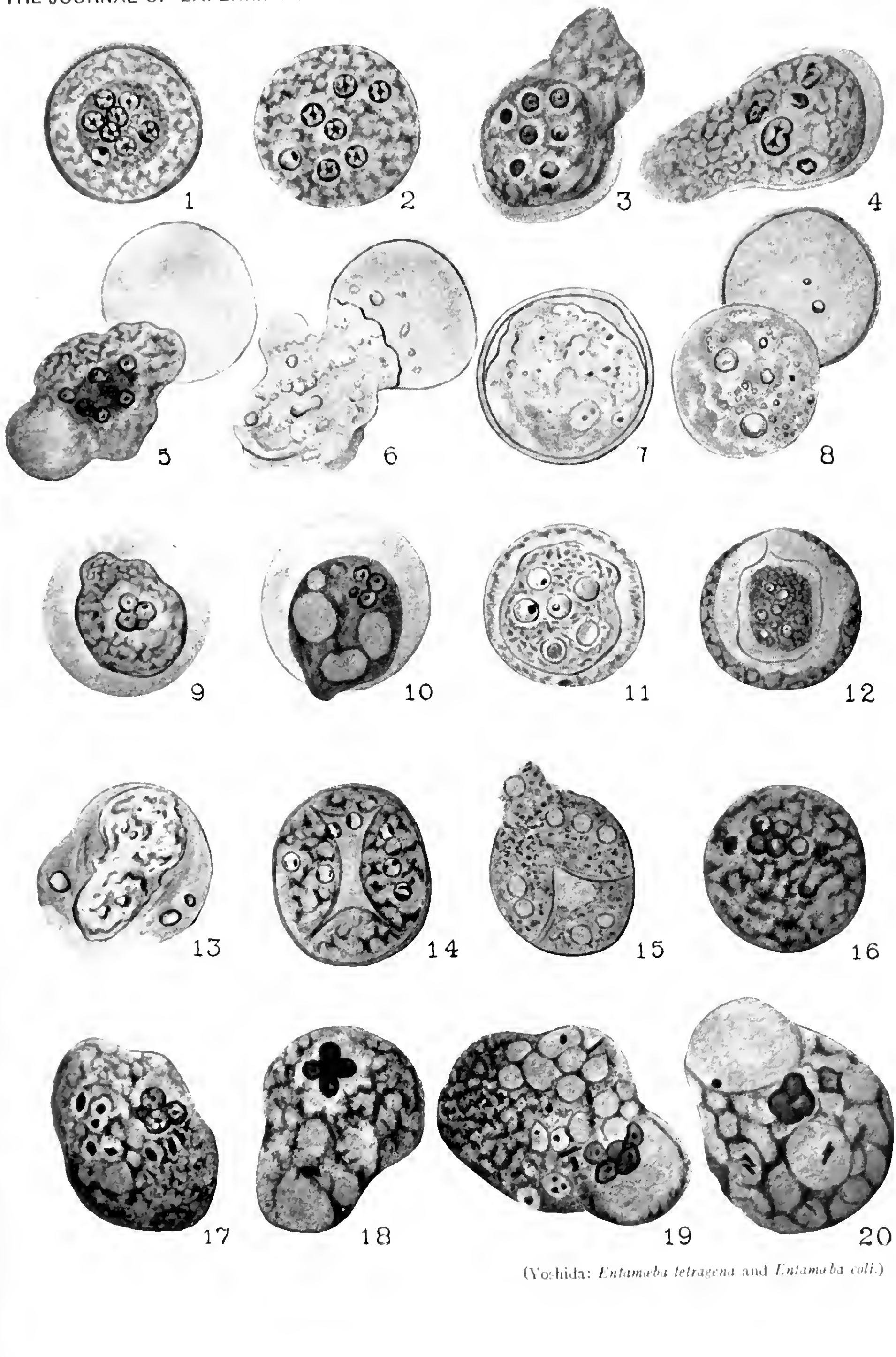
18 to 21. Cyst formation of atypical forms. Multiple nuclear division within the nuclear substance.

22 to 25. Cyst formation of atypical forms. Multiple nuclear division outside the degenerating nucleus.

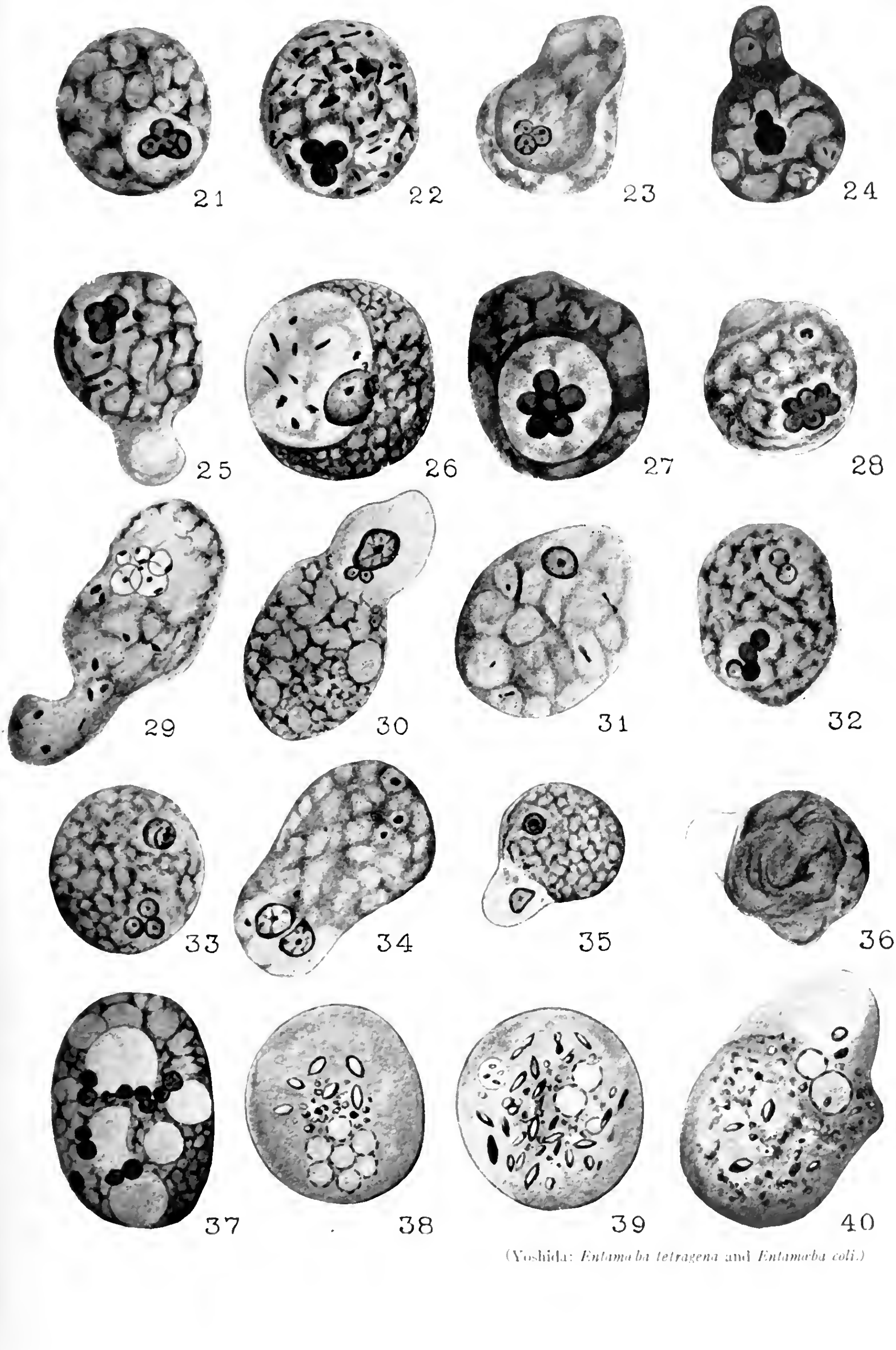
26 and 27. Degenerated atypical forms.

28 and 29. Reproduction: 28, by division; 29, by a form of budding.

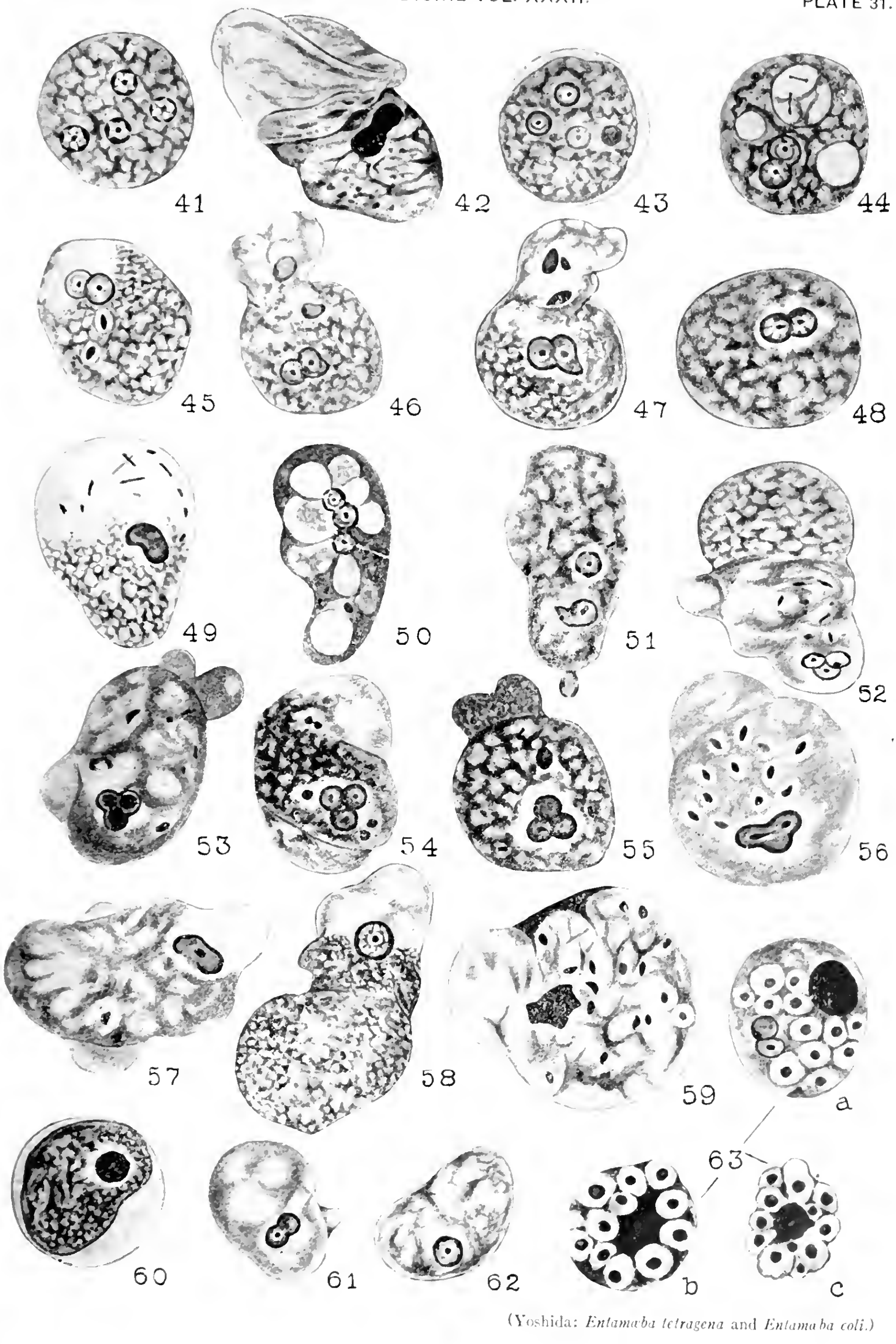
980



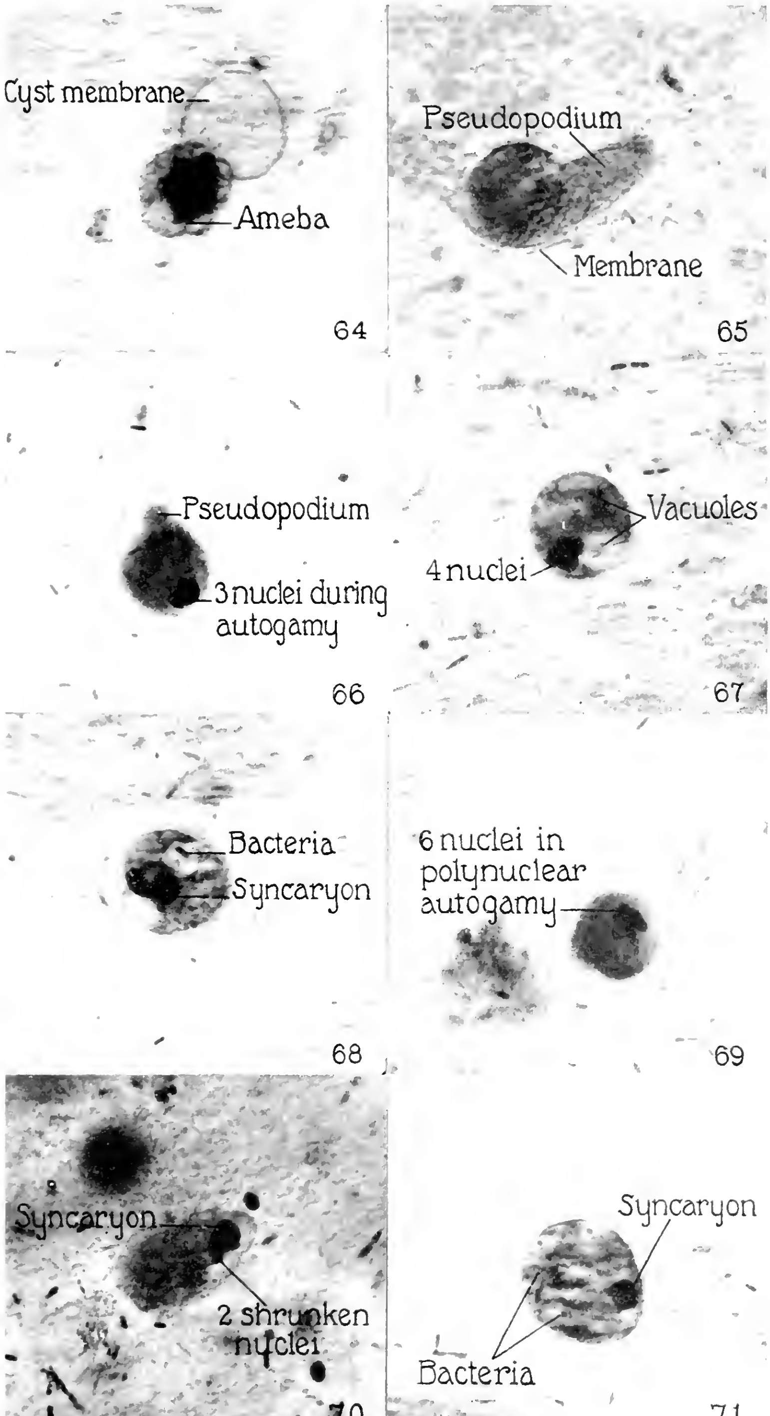
(Yoshida: *Entamœba tetragena* and *Entamœba coli*.)



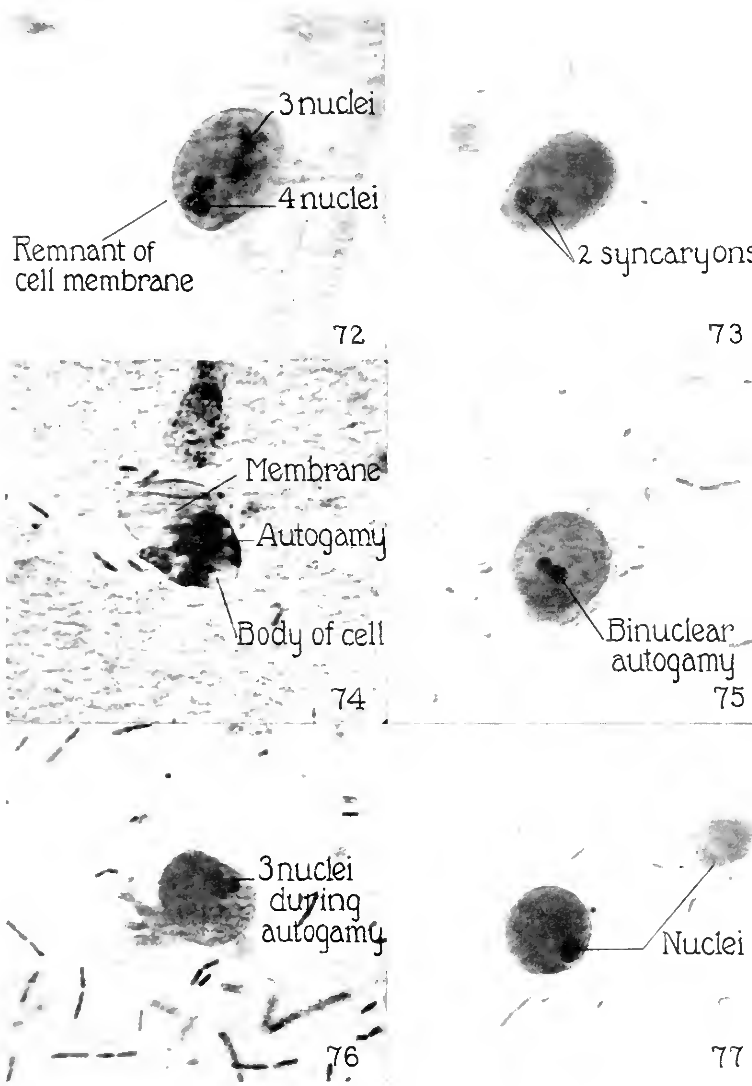
(Yoshida: *Entamœba tetragena* and *Entamœba coli*.)



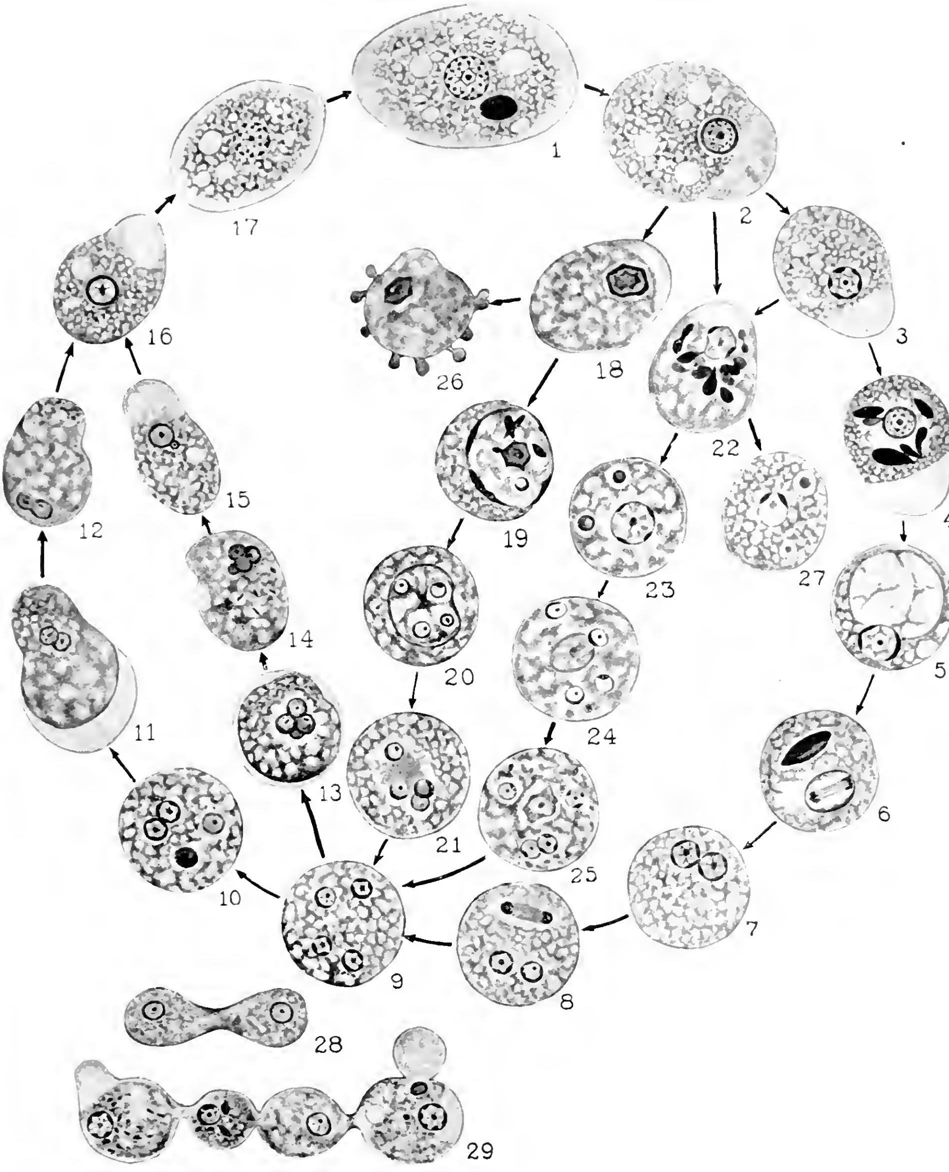
(Yoshida: *Entamœba tetragena* and *Entamœba coli*.)



(Yoshida: *Entamœba tetragena* and *Entamœba coli*.)



(Yoshida: *Entamœba tetragena* and *Entamœba coli*.)



(Yoshida: *Entamœba tetragena* and *Entamœba coli*.)

ETIOLOGY OF YELLOW FEVER.

XII. Chemotherapy versus Serotherapy in Experimental Infection with Leptospira icteroides.

By HIDEYO NOGUCHI, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, April 3, 1920.)

The work of Ehrlich and Hata[1] on the therapeutic effects of arsenical organic compounds in various infections caused by spirochetal or protozoan organisms has led to considerable investigation by later workers, notably Jacobs, Heidelberger, Amoss, and Bull[2-5] and Pearce and Brown,[6] of the extent to which this kind of curative method is applicable. The present experiments were undertaken, not as research in chemotherapy, but merely as part of a routine study of experimental infection in guinea pigs with *Leptospira icteroides*, derived from certain cases of yellow fever.[7] It was of particular interest to ascertain how *Leptospira icteroides* would behave toward the two widely employed chemotherapeutic agents, salvarsan and neosalvarsan, and what difference, if any, there is between its behavior and that of the inciting agent of infectious jaundice in this respect, for the latter has been extensively studied by Inada, Ido, and their collaborators,[8] as well as

[1] Ehrlich, P., and Hata, S., Die experimentelle Chemotherapie der Spirillosen, Berlin, 1910.

[2] Jacobs, W. A., *J. Exp. Med.*, 1916, xxiii, 563.

[3] Jacobs, W. A., Heidelberger, M., and Amoss, H. L., *J. Exp. Med.*, 1916, xxiii, 569.

[4] Jacobs, W. A., Heidelberger, M., and Bull, C. G., *J. Exp. Med.*, 1916, xxiii, 577.

[5] Jacobs, W. A., and Heidelberger, M., *J. Biol. Chem.*, 1915, xx, 513, 659, 685; xxi, 103, 145, 403, 439, 455, 465.

[6] Pearce, L., and Brown, W. H., *J. Exp. Med.*, 1918, xxviii, 109.

[7] Noguchi, H., *J. Exp. Med.*, 1919, xxix, 547, 565, 585; xxx, 1, 9, 13, 87, 95, 401; 1920, xxxi, 135, 159.

[8] Inada, R., Ido, Y., Hoki, R., Kaneko, R., and Ito, H., *J. Exp. Med.*, 1916, xxiii, 377. Kaneko, R., and Okuda, K., *Verhandl. japan. path. Ges.*, 1916, vi, 49.

by European investigators, especially Uhlenhuth and Fromme.[9] A large number of experiments with salvarsan and neosalvarsan, atoxyl, and arsacetin against *Leptospira icterohæmorrhagiæ* was also carried out in 1917, with negative results.[10] Being regarded by some as a spirochete, *Leptospira icterohæmorrhagiæ* was expected to yield to chemotherapy by means of salvarsan or its derivative, but Inada and Ido and others soon found that neither salvarsan nor neosalvarsan had any definite therapeutic value in infections with this organism. In this respect, at least, the organism discovered by Inada and Ido in infectious jaundice did not behave like the other pathogenic spirochetes, and this characteristic may be regarded as giving further support to the opinion that the leptospira group of organisms forms a type of its own and differs from the other pathogenic genera of Spirochætoidea. The experiments here reported concern the behavior of *Leptospira icteroides* toward the arsenical compounds, not only in the animal body, but also *in vitro*, and the effect upon the organism of salvarsanized serum. From the practical standpoint it seemed advisable to include also a brief comparison of the action of the arsenical preparations and that of anti-*icteroides* immune serum upon *Leptospira icteroides* in experimental infection and *in vitro*.

Effect of Salvarsan and Neosalvarsan upon the Course of Experimental Infection of Guinea Pigs Inoculated with Leptospira icteroides.

Several series of experiments were performed in order to ascertain whether administration of salvarsan or neosalvarsan simultaneously with the inoculation or shortly afterwards would in any way influence the development of the experimental leptospiral infection in guinea pigs. If the virulence of *Leptospira icteroides* for this animal were constant a few series of experiments only would have been sufficient to determine the point, but owing to the variable character of the pathogenicity of the organism for individual guinea pigs it was necessary to repeat similar experiments. In some series control animals survived or escaped infection, hence the interpretation of the effect of the drugs was rendered inconclusive. In the earlier experiments

[9] Uhlenhuth and Fromme, *Z. Immunitätsforsch., Orig.*, 1916, xxv, 418.
[10] Noguchi, H., unpublished results.

the amounts of infecting material were near a single lethal dose, even subminimum lethal doses being used; *i.e.*, a quantity capable of producing in the majority of instances a mild infection with recovery. The mode of experiment was practically the same in all the series. In one instance an unneutralized solution of salvarsan was employed, otherwise salvarsan has been used as an alkaline solution. The injection of the infecting material was intraperitoneal and that of the drugs subcutaneous.

TABLE I.

Inoculation of Infecting Material into Guinea Pigs Followed by Injection of Drug.

November 27, 1919. The amount of infecting material used was 0.5 cc.

Amount of arsaminol-natrium.	Course of disease.	Result.	Remarks.
gm.			
0.03	Mild; 6 to 8 days.	Recovered.	Died in 15 days; no jaundice.
0.02	Moderate (+); 6 to 11 days.	"	No jaundice.
0.01	Severe (++++); 6 to 11 days.	"	Died in 13 days; jaundice fading.
0.005	Mild; 7 to 9 days.	"	No jaundice.
0.0025	Severe (++++); 6 to 11 days.	"	Killed in 11 days; typical lesions.
0.001	Died in 6 days.	Typical.	
0.0005	" " 9 "	"	
Control.	Severe (+++); killed in 6 days.	"	
"	Moderate (++); 7 to 8 days.	Recovered.	
"	Mild (±); 7 days.	"	

Series 1.—November 27, 1919. The infecting material consisted of a mixture of citrate blood (showing the leptospiras) of a guinea pig experimentally infected with Guayaquil Strain 1 and a rich culture of the same strain. Ten guinea pigs were inoculated intraperitoneally, each with 0.5 cc. of the mixture, and all but three (controls) then received subcutaneously a solution of arsaminolnatrium (a Japanese preparation of neosalvarsan), the quantities given ranging from 0.03 to 0.0005 gm. (Table I).

The amount of infecting material used in this series was near a single minimum lethal dose, as shown in the control animals and also in those which received the smallest amounts of the drug. It is noteworthy that in the three guinea pigs which received 0.03, 0.02,

and 0.005 gm. of the drug, respectively, there was no jaundice at any period of the infection.

Series 2.—December 3, 1919. The infecting material consisted of a mixture of 1 cc. each of cultures of Guayaquil Strains 1 and 5, and 7 cc. of heart's blood from a guinea pig infected typically with Strain 1. 0.5 cc. of the mixture was inoculated intraperitoneally into each of sixteen guinea pigs. One group of eight animals was then inoculated subcutaneously with an unneutralized solution of salvarsan and the other group of eight with the solution of neosalvarsan in doses

TABLE II.

Inoculation of Infecting Material into Guinea Pigs Followed by Injection of Drug.

December 3, 1919. The amount of infecting material used for the treated animals was 0.5 cc.

Treated animals.			Untreated controls.	
Amount of drug.	Course of disease.		Test for virulence of infecting material used.	Course of disease.
	Salvarsan (acid solution).	Neosalvarsan.		
gm.			*cc.*	
0.03	Mild; recovery.	Mild; recovery.	1.0	Severe (++++); recovery.
0.02	No symptoms.	Severe (+++); recovery.	0.5	Mild; recovery.
			0.2	Moderate (++); recovery.
0.01	" "	Mild; recovery.		
0.005	" "	No symptoms.	0.1	No symptoms.
0.002	Died in 12 days; typical.	" "		
0.001	No symptoms.	" "		
0.0005	" "	Died in 13 days; typical.		
0.0002	" "	Severe (+++); recovery.		

varying from 0.03 to 0.0002 gm. There were four control guinea pigs which received varying doses of the infecting material and were not treated with the drug (Table II).

The results of these experiments suggest a possible slight protective effect of the drugs in some individuals, although, from the mildness of the infection in the control animals the survival of those individuals may also be explained on the basis of a natural resistance to *Lepto-*

spira icteroides. It is of interest to note that more guinea pigs among those treated with salvarsan escaped the infection than among those treated with neosalvarsan. The indecisive character of the experiments made necessary another series with multiple minimum lethal doses. In Series 3 at least 50 minimum lethal doses were used for each guinea pig.

Series 3.—March 13, 1920. The infecting material consisted of a mixture of 5 cc. of a culture of Guayaquil Strain 1, 5 cc. of kidney emulsion, and 5 cc. of citrated heart's blood from a guinea pig inoculated with the same strain 4 days previously. In order to produce a fatal infection in guinea pigs 0.5 cc. of the mixture was inoculated intraperitoneally into each animal. As the protocol shows, 0.01 cc. of this material killed a control animal in 7 days with typical symptoms, and a much smaller quantity would have been sufficient to cause a fatal infection, although the minimum lethal dose was not determined in the present series of experiments.

Salvarsan was dissolved in sterile distilled water and a 0.1 N sodium hydroxide solution was gradually added until the precipitate first formed was completely redissolved. The final concentration of the drug was made 0.1 gm. per 10 cc. (stock solution). Neosalvarsan was dissolved in sterile distilled water in the ratio of 0.1 gm. per 10 cc. (stock solution). Further dilutions were made with 0.9 per cent salt solution.

The animals were inoculated with 0.5 cc. of the infecting material intraperitoneally, and within about 30 minutes varying amounts of salvarsan and neosalvarsan solution were injected subcutaneously. The amounts of the drug were 0.00005, 0.0001, 0.0002, 0.0005, 0.001, 0.002, and 0.003 gm. for guinea pigs of 350 gm., and each dose was contained in from 0.5 to 3 cc. of fluid, according to convenience in measurement. Table III gives the results.

Series 4.—March 18, 1920. Although it was still too early to know the results of the series of March 13, another series was begun on March 18 in which larger doses (0.01, 0.02, and 0.03 gm.) of salvarsan and neosalvarsan were used. The solutions of the drugs were freshly prepared from another set of ampules, and the infecting material consisted of a guinea pig kidney emulsion of Guayaquil Strain 1 of *Leptospira icteroides*. Other technical details were the same as in the previous experiments. Table IV gives the results.

The experiments of Series 3 (March 13) show that guinea pigs receiving at least 50 minimum lethal doses of a strain of *Leptospira icteroides* intraperitoneally and varying quantities of salvarsan or neosalvarsan subcutaneously within 30 minutes from the time of inoculation of *icteroides* undergo a typical course of leptospira infection, resulting in the majority of instances in death within a

period the variations of which may be considered usual in such a series of experiments. With salvarsan there were two instances of recovery after a severe infection coinciding with elevated doses of the drug (0.001 and 0.003 gm.), while in animals receiving less of the

TABLE III.

Inoculation of Infecting Material into Guinea Pigs Followed by Injection of Drug.

March 13, 1920. The amount of infecting material used for the treated animals was 0.5 cc.

Treated animals.			Untreated controls.	
Amount of drug.	Course of disease.		Test for virulence of infecting material used.	Course of disease.
	Salvarsan.	Neosalvarsan.		
gm.			*cc.*	
0.00005	Died in 5 days; typical.	Recovery after a mild infection, with definite jaundice from Mar. 20–24, 1920.	0.5 0.1	Died in 6 days; typical. Died in 7 days; typical.
0.0001	" " 8 " "	Died in 5 days; typical.	0.01	Died in 7 days; typical.
0.0002	" " 5 " "	" " 6 " "		
0.0005	" " 6 " "	" " 7 " "		
0.001	Recovery after a typical infection, with intense jaundice from Mar. 19–25, 1920.	" " 9 " "		
0.002	Died in 6 days; typical.	Recovery after a mild infection, with jaundice from Mar. 19–23, 1920.		
0.003	Recovery after a typical infection lasting from Mar. 18–20, 1920.	Recovery after a severe infection, with intense jaundice from Mar. 18–23, 1920.		

drug death occurred in two instances 2 days earlier than in controls, a fact worthy of notice. With neosalvarsan the number of recovered animals was three, two with the largest doses of the drug (0.002 and 0.003 gm.) and one with the minutest dose employed (0.00005 gm.).

All the rest died in from 5 to 9 days with typical symptoms. The results both with salvarsan and with neosalvarsan suggest that these arsenical compounds, when employed in certain quantities, may somewhat modify the severity of the infection and occasionally save a guinea pig from death, although failing to suppress the infection completely.

The results of the experiments of Series 4 (March 18) were less favorable than those of Series 3. Here, of the two controls one died in 12 days and the other recovered after a definite infection. Two

TABLE IV.

Inoculation of Infecting Material into Guinea Pigs Followed by Injection of Drug.

March 18, 1920. The amount of infecting material used for the treated animals was 0.5 cc.

Treated animals.			Untreated controls.	
Amount of drug.	Course of disease.		Test for virulence of infecting material used.	Course of disease.
	Salvarsan.	Neosalvarsan.		
gm.			*cc.*	
0.01	Died in 11 days; typical.	Died in 12 days; typical.	0.5	Died in 12 days; typical.
0.02	Had fever but no jaundice; recovery.	" " 12 " "	0.5	Recovery after a definite infection, with intense jaundice from Mar. 30–31, 1920.
0.03	Died in 11 days; typical.	Had fever but no jaundice; recovery.		

of the three guinea pigs treated, either with salvarsan or neosalvarsan, died in from 11 to 12 days, and the one animal which survived in each set had fever indicative of an abortive leptospira infection.

From the therapeutic standpoint neither salvarsan nor neosalvarsan is of any value in experimental infections of guinea pigs with *Leptospira icteroides*. In contrast, it may be interesting to include here a protocol illustrating the highly specific protective value of an anti-*icteroides* serum (horse). The amount of the immune serum required to protect a guinea pig from an infection with *Leptospira icteroides* is exceedingly minute.

Contrasted Effect of Anti-icteroides Immune Serum in Vivo.

March 11, 1920. The experiments with the immune serum were undertaken with the same strain of *Leptospira icteroides* (Guayaquil No. 1) that was used in the chemotherapeutic experiments already described. The material was a mixture of the emulsions of kidney and liver from a guinea pig which was showing early symptoms of the leptospira infection. As the protocol shows, the minimum lethal dose of this emulsion was such that 1 cc. of a 1:10,000 dilution of it killed a guinea pig with typical symptoms in 12 days, and the same quantity of a 1:1,000 or 1:100 in 10 days. 0.5 cc. of the original emulsion killed a guinea pig in 8 days.

TABLE V.

Inoculation of Infecting Material into Guinea Pigs Followed by Injection of Anti-icteroides Serum.

March 11, 1920. The amount of infecting material used for the treated animals was 0.5 cc.

Treated animals.			Untreated controls.	
Anti-*icteroides* immune serum.		Course of disease.	Test for virulence of infecting material used.	Course of disease.
Amount.	Dilution.			
cc.			*cc.*	
1	1:100,000	Recovery after a mild infection.	0.5	Died in 8 days; typical.
			0.1	Recovery after a severe infection (exceptional resistance).
1	1:10,000	No symptoms.		
1	1:1,000	" " (suspicion of trace of jaundice on Mar. 20, which had disappeared the following morning).	0.01	Died in 10 days; typical
			0.001	" " 10 " "
			0.0001	" " 12 " "
			0.00001	No symptoms.
1	1:100	No symptoms.	0.000001	" "
1	1:10	" "		

For infecting guinea pigs to be treated with anti-*icteroides* serum, 0.5 cc. (about 5,000 minimum lethal doses) of the same emulsion was intraperitoneally injected. The different amounts of the anti-*icteroides* serum (collected from Horse 2 on February 25, 1920) were then injected, also intraperitoneally, within half an hour after the inoculation. The amounts of immune serum were 0.00001, 0.0001, 0.001, 0.01, and 0.1 cc. Table V is a record of the results.

According to a conservative estimate, therefore, the power of the immune serum is such that 1 cc. of a 1: 10,000 dilution prevented an infection when the dose of the infecting material was 5,000 minimum

lethal doses. In other words, 1 cc. of the serum had the power to protect a guinea pig of 350 gm. body weight against 50,000,000 (10,000 × 5,000) minimum lethal doses when administered intraperitoneally 30 minutes after intraperitoneal inoculation. The specific protective property of the immune serum is indisputably highly efficacious as compared with salvarsan or neosalvarsan, the value of which is at least doubtful. A point of considerable importance is that in certain guinea pigs receiving small quantities of salvarsan and neosalvarsan the period before death seemed to be shortened by 2 days (5 days), as compared with the average period in untreated control animals (7 days). It may be that the predilection of arsenic compounds for the renal tissues had a definite predisposing effect, due to chemical injury, upon this organ, which *Leptospira icteroides* also attacks particularly.

Direct Action of Salvarsan and Neosalvarsan upon Leptospira icteroides.

Ehrlich makes a special point of having found, in his quest for chemotherapeutic agents, that a preparation whose destructive power on a microbic organism is great *in vitro* may have no, or only a slight antagonistic effect when introduced into the animal body, or the relation between the effect manifested *in vitro* and *in vivo* may be the reverse. Ehrlich's efforts to find a chemotherapeutic preparation were principally directed toward its effect *in vivo*, since the pathogenic parasites with which he was working belonged to the class of protozoa or a class closely allied to it, and there were no virulent cultures on hand to be tested *in vitro* as well as *in vivo*. Certain arsenic compounds elaborated by Ehrlich and his coworkers displayed a highly sterilizing effect on various spirochetoid organisms when introduced into the animal body infected with them, while the direct effect of these preparations upon the same organisms *in vitro* was almost nil. Ehrlich interpreted the highly parasiticidal properties of these compounds as due to a certain modification (reduction) in the animal body of substances otherwise comparatively inert. Salvarsan and *Treponema pallidum* constitute a good example in point.

Leptospira icteroides is resistant to saponin,[11] a property which alone would serve to differentiate it from certain other groups of spiro-

[11] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 13.

chetoid organisms (*Treponema* and *Spironema*). The fact that *in vivo* the leptospiras were scarcely influenced by the introduction of salvarsan or neosalvarsan constituted another point of dissimilarity between the leptospira and other spirochetal parasites. Hence the effect of salvarsan or neosalvarsan upon the leptospira from yellow fever cases *in vitro* presented a further interesting subject for study. Several experiments were also performed to determine the behavior of blood serum derived from a rabbit which had been injected intravenously 1 hour previously with salvarsan or neosalvarsan in considerable quantities; that is, a study was made of the effect of salvarsanized serum, as well as of drug solutions, upon *icteroides in vitro*.

TABLE VI.

Addition of Drug to Culture of Leptospira icteroides.

November 26, 1919.

Final concentration of arsaminolnatrium.*	Result.
1:2,000	All inactive and most have disappeared.
1:6,000	" " " some degenerated.
1:20,000	" "
1:60,000	" " thinner, and smoother.
1:200,000	" "
1:600,000	For the most part inactive.
1:2,000,000	" " " " " some still active.
Water (control).	" " " " active, a few inactive.

* The concentration resulting from the mixture of the drug solutions with the culture is given.

To each of several tubes containing 1 cc. of a culture of Guayaquil Strain 6 was added 1 cc. of different dilutions of salvarsan, neosalvarsan, or arsaminolnatrium. The mixtures were allowed to stand at room temperature and examined after 2 or 2½ hours, and again after 24 or 48 hours. As Tables VI and VII show, sufficient quantities of the drugs rendered the leptospiras immotile, and brought about general disintegration. The highest dilution of any of the drugs which still killed the organism was about 1:200,000.

In testing the leptospiricidal strength of the solutions of salvarsan or neosalvarsan, it was important to take note of the reactions of the solutions. Salvarsan, according to the usual practice, is first treated with sodium hydroxide solution until completely precipitated out

at the point of neutral reaction. In order to obtain a clear solution it is necessary to add more alkali until the precipitate completely dissolves. At this point the solution is no longer slightly, but intensely alkaline. By reducing the alkalinity with hydrochloric acid to a reaction of about pH 8 a bulky precipitate is once more formed, and at pH 7 the entire substance flocculates out of the solution. In making a microbicidal titration a clear alkaline solution was used in ascending dilutions to eliminate the destructive effect of the reaction alone. It was found that the 1: 1,000 dilution was still too strongly

TABLE VII.

Addition of Drugs to Culture of Leptospira icteroides.

December 3, 1919.

Final concentration of the drug.	Salvarsan.		Neosalvarsan.	
	2½ hrs.	24 hrs.	2½ hrs.	24 hrs.
1:200	Precipitate (?).	Precipitate heavy.	All inactive.	All immobilized.
1:2,000	Some active; precipitate.	All immobile; precipitate.	" "	" "
1:20,000	Some active; precipitate.	All immobile; precipitate.	" "	" "
1:200,000	For the most part active.	All immobile.	" "	" "
1:2,000,000	For the most part active.	For the most part immobile; few active.	For the most part active.	Few active.
Saline (control).	All active.	All active.	All active.	For the most part active.

alkaline to be used (far beyond pH 8), the leptospiras dying rapidly in such a solution, while dilutions higher than 1:10,000 had a reaction which was practically that of the diluent (saline solution), pH 7. Neosalvarsan dissolved in distilled water in 1:100 dilution is a clear amber-yellow and shows to phenol red an intense red color; in diluting to 1:1,000 with saline solution its reaction approaches pH 7.2; in 1:10,000 dilution it is no longer perceptible to phenol red. In actual experiments, however, 1 cc. of each of the dilutions of the drugs was mixed with 1 cc. of a rich culture of one of the *icteroides* strains which

had a reaction of pH 7.4. This mixing brought down the pH value of the lower dilutions and raised that of the higher. The reactions between pH 7 and pH 7.8 are well borne by *icteroides*, the optimum being near pH 7.2 to 7.4. A reaction beyond pH 8 or below pH 6.6 is unsuitable for the existence of the organism. The devitalizing action of salvarsan and neosalvarsan, even in optimum reactions, is rather slow, a contact of many hours being required before death ensues, as the protocol shows.

TABLE VIII.

Addition of Drugs to Culture of Leptospira icteroides.

March 18, 1920.

Final concentration of the drug.	Salvarsan.			Neosalvarsan.		
	Reaction.	After 15 min.	After 18 hrs.	Reaction.	After 15 min.	After 18 hrs.
1:200	Intensely alkaline.	Dead.		Slightly over pH 8.	Active.	Dead.
1:2,000	Intensely alkaline.	"		pH 7.2	"	"
1:20,000	pH 7.2	Active.	Dead.	pH 7.2	"	"
1:200,000	pH 7.2	"	"	pH 7.2	"	"
1:2,000,000	pH 7.2	"	" (?).	pH 7.2	"	Active.*
Saline control (no drug).	pH 7.2	"	Active.	pH 7.2	"	"

* All found dead after 96 hours; the control tube was lost through contamination.

To each of several tubes containing 1 cc. of the same culture of *icteroides*, Guayaquil Strain 5 (pH 7.4), was added 1 cc. of several different dilutions of drugs, 1:100, 1:1,000, 1:10,000, 1:100,000, and 1:1,000,000 of alkalinized salvarsan solution and neosalvarsan. The mixtures were allowed to stand at 26°C. and the contents examined under the dark-field microscope after 15 minutes and again after 18 hours. The results are recorded in Table VIII.

It is evident that *Leptospira icteroides* is highly sensitive to the action of salvarsan and neosalvarsan, but their action is comparatively slow, requiring many hours contact. The effects of the drugs in a culture medium are found to be approximately the same as in the case of direct mixing of culture and solutions.

Various dilutions of the drugs were added to the usual medium (rabbit serum, 25 per cent, agar, 0.3 per cent, total volume, 6 cc.). The culture used was Guayaquil Strain 1, and the tubes were allowed to stand for 5 days at room temperature (26°C.). A medium containing salvarsan or neosalvarsan in a ratio of more than 1:200,000 was found to be unsuitable for the growth of the organism (Table IX). No attempt was made to determine quantitatively the exact leptospiricidal titers of the two drugs.

TABLE IX.

*Addition of Drugs to Culture Media.**

March 6, 1920.

Final concentration of the drug.	Salvarsan.	Neosalvarsan.
1:600	−	−
1:1,800	−	−
1:6,000	−	−
1:18,000	−	−
1:60,000	−	−
1:180,000	−	−
1:600,000	+	<+
1:1,800,000	+	+
1:6,000,000	+	+
Control.	+	+
"	+	+

* The medium consisted of rabbit serum, 25 per cent, agar, 0.3 per cent, total volume, 6 cc.

The changes in the color of salvarsan, when observed after 12 days standing, were noticeable in the tubes containing dilutions from 1:100,000 up, while with neosalvarsan the changes were not found in dilutions below 1:10,000.

Effect of Salvarsanized and Neosalvarsanized Serum.

The problem here was to determine the effect of the living body upon the drugs when the latter were introduced into the blood circulation. It has been assumed that in the animal or human body they are converted into highly spirocheticidal substances, as is said to be the case with respect to the organisms of syphilis, yaws, and relapsing fevers. It has already been shown that these drugs are practically without effect *in vivo* upon the course of the *icteroides* infection, and that they are highly destructive to the organism *in vitro*. It was this discrepancy which suggested the study of the salvarsanized serum.

The mode of experiment was to inject a rabbit intravenously with salvarsan or neosalvarsan in a ratio of 0.05 gm. per kilo of body weight, the blood being drawn 1 hour after the time of injection. Two rabbits, weighing 1,500 and 2,000 gm. respectively, were injected on March 18, 1920, intravenously, one with salvarsan (alkaline), the other with neosalvarsan. After 1 hour they were killed for the blood. The clear serums were collected the next day and used in the active state in order to determine whether they were in any way different from a normal rabbit serum when mixed *in vitro* with the same quantity (1 cc. in each case) of a rich culture of Guayaquil Strain 5. The mixtures were kept in a thermostat at 28°C. during the period of observation. Table X gives the results.

The experiments demonstrated clearly the difference between the normal and the salvarsanized rabbit serums. All the leptospiras mixed with the latter died within 72 hours, while those in normal rabbit

TABLE X.

Effect of Salvarsanized, Neosalvarsanized, and Normal Rabbit Serum in Vitro.

March 18, 1920.

Duration of contact.	Salvarsanized rabbit serum, 1 cc., plus culture, 1 cc.	Neosalvarsanized rabbit serum, 1 cc., plus culture, 1 cc.	Normal rabbit serum, 1 cc., plus cultuie, 1 cc.
hrs.			
1	All active.	All active.	All active.
18	For the most part motile, but sluggish.	For the most part sluggish.	" "
48	No observation.	No observation.	No observation.
72	All dead and degenerated.	All dead and degenerated.	All active and multiplying.

serum steadily multiplied. It may be mentioned that the reactions of the drugged serums as well as the reaction of the normal serum were pH 7.4, hence the question of hydrogen ion concentration does not enter into the present comparative study. At the end of 18 hours the organisms were already less active in the drugged serums than in the normal serum.

It occurred to me that the cause of this slow leptospiricidal action of the salvarsanized and neosalvarsanized serums might be due to a gradual development of injurious substances by slow oxidation of the drugs. If this were the case we should find in these tubes a powerful and rapidly acting leptospiricidal substance at the end of 72 hours of exposure to the same experimental conditions. Two different experiments were carried out to ascertain this point.

In the first experiment 0.5 cc. of a rich culture of the Merida strain[12] of *icteroides* was added to each of the three tubes containing salvarsanized, neosalvarsanized, and normal serum respectively. The results were similar to those observed with the Strain 5 culture; that is, there was no effect upon the organisms during the 1st hour; they were all active at the end of that time. After 24 hours the leptospiras in the salvarsanized serum were all dead, but there were many active survivors in the neosalvarsanized serum. After 48 hours, however, they were for the most part dead in the neosalvarsanized serum, but all were active in the tube containing the normal serum. In the second experiment three tubes containing 1 cc. of each of the serums were placed at 28°C. for 72 hours, then 1 cc. of the rich Merida culture was added to each. The mixtures were again put at 28°C. for the period of observation. Table XI gives the results of this experiment.

TABLE XI.

*Effect on Leptospira icteroides (Merida Culture) of Salvarsanized, Neosalvarsanized, and Normal Rabbit Serums after the Serums Had Stood for 72 Hours in the Incubator at 28°C.**

Experiment of Mar. 22, 1920.	Salvarsanized serum, 1 cc., plus culture, 1 cc.	Neosalvarsanized serum, 1 cc., plus culture, 1 cc.	Normal serum, 1 cc., plus culture, 1 cc.
After 1 hr.	All active.	All active.	All active.
" 18 hrs.	Some active.	Many active.	" "
" 48 "	All dead.	All dead.	" "

* There was a slight shift of pH value (to pH 7.8) in these serums on standing, but they were brought back to pH 7.6 by the addition of the culture.

These two series of experiments indicate that a certain antagonistic substance seems to have developed in the tubes containing the salvarsanized and neosalvarsanized serums during the period of 72 hours at 28°C., as shown by an earlier death and degeneration of the leptospiras than was the case with the fresh samples of these serums. The difference was especially definite in the effects observed at the end of 18 hours with the first series. On the other hand, in no instance was there any rapid immobilization or destruction of the organisms, the drugs having exerted no perceptible effect upon them even after an hour's contact. At all events, no rapidly leptospiricidal substances could be demonstrated in the salvarsanized or neosalvarsanized rabbit serum after exposure to the air for 72 hours at 28°C. The death of the leptospiras in these drugged serums was slow but certain.

[12] This strain was isolated from a case of yellow fever in Merida, Mexico, and will be described in a later paper (Noguchi, H., and Kligler, I. J., *J. Exp. Med.*, 1920, xxxii, in press).

The question arises as to the form in which salvarsan or neosalvarsan existed in the blood serum of these two rabbits. This we do not know, but whatever its state, its ultimate concentration must correspond at most with a dilution of 1:20,000; that is, on the assumption that 0.05 gm. of the compound had diffused out in a space of 1,000 cc. In reality, the volume representing 1 kilo of body weight of the rabbit must be considerably smaller than 1,000 cc., hence the concentration of the drugs in the serum must have been stronger than 1:20,000. Other experiments (Table VII) showed that salvarsan or neosalvarsan added directly to a rich culture kills the latter within 24 hours; that is, in less time than that required with the salvarsanized or neosalvarsanized serum, which was at least 48 hours. Perhaps the arsenic compounds had undergone a modification in the animal body which converted them into substances operating much more slowly. As the animals did not urinate after the injection of the drugs up to the time of collecting the serum the slowness of action cannot be explained by elimination of the drug through the urine. Moreover, a dilution of 1:200,000 of the drugs when added directly to a culture caused the death of the latter in 18 hours. To summarize, then, serum drawn from rabbits at the end of 1 hour from the time of an intravenous injection of salvarsan or neosalvarsan in a ratio of 0.05 gm. per kilo of body weight possesses a slowly acting leptospiricidal property, which does not seem to be much increased in respect to rate of action by an exposure to the air for a period of 72 hours at 28°C.

Contrasted Effect of Anti-icteroides Immune Serum in Vitro.

Comparison has already been made of the chemotherapeutic value of salvarsan and neosalvarsan and the serotherapeutic value of anti-*icteroides* immune horse serum, showing the comparative inefficacy of these drugs and the highly potent specific protective property of the serum. Since the leptospiricidal power of the drugs was considerable *in vitro*, in sharp contrast with their lack of perceptible protective action *in vivo*, a similar comparison of the action *in vitro* of the immune serum and that of the drugs was of practical interest.

The same immune serum which was used in the experiments recorded in Table V was mixed with 1 cc. of a rich culture of Guayaquil Strain 5, the object being

to determine how high a dilution of the serum still had leptospiricidal power in the test-tube. The maximum dilution which caused complete immobilization and subsequent degeneration within 1 hour was 1:20, while a 1:200 dilution caused considerable but incomplete agglutination and degeneration within 18 hours. No effect whatever was perceptible in a mixture containing the serum in a dilution of 1:1,000 or less. The results of the experiment are recorded in Table XII.

When we place side by side this low titer *in vitro* of the immune serum (1 cc. of a 1:100 dilution to 1 cc. of culture) and its protective titer *in vivo* (1 cc. of a 1:10,000 dilution to 0.5 cc. of culture, or 5,000 minimum lethal doses), it is easy to conceive at once how completely

TABLE XII.

Effect of Anti-icteroides Immune Serum upon Leptospira icteroides in Vitro.

March 19, 1920.

Anti-*icteroides* immune serum.		After 1 hr.	After 18 hrs.
Amount.	Dilution.		
cc.			
1	1:10 (final dilution 1:20).	All dead.	All degenerated.
1	1:100 (" " 1:200).	For the most part active.	For the most part agglutinated and degenerated, but some still motile.
1	1:1,000 (" " 1:2,000).	All active.	All active.
1	1:10,000 (" " 1:20,000).	" "	" "
1	1:100,000 (" " 1:200,000).	" "	" "
1	Saline control.	" "	" "

reverse is the relation that exists between the behavior of the immune serum on the one hand and that of salvarsan and neosalvarsan on the other toward *Leptospira icteroides in vitro* and *in vivo*.

SUMMARY AND CONCLUSIONS.

In several series of experiments guinea pigs were variously infected with different amounts of *Leptospira icteroides*, either in the form of culture, organ emulsion from infected guinea pigs, or a mixture of both. The infecting materials were of different grades of virulence; in some series the amount given was near a single lethal dose, in others a subminimum lethal dose was given, *i.e.* causing mild infection

with recovery in the majority of animals, and in still others the animals were injected with at least 50 minimum lethal doses of a mixture of a culture and a highly virulent organ emulsion from a guinea pig. The animals were inoculated intraperitoneally, and within about 30 minutes each was injected subcutaneously with a different amount of salvarsan or neosalvarsan. The amounts injected were in most series 0.0005, 0.001, 0.002, 0.005, 0.01, 0.02, and 0.03 gm. per 350 to 450 gm. of body weight, and in one series, in addition to this dosage, 0.00005, 0.0001, and 0.0002 gm. were also tried.

Among the guinea pigs treated either with salvarsan or with neosalvarsan there were more recoveries than among the controls, but they were not in strict proportion to the amounts of the drugs injected. In the experiments with 50 minimum lethal doses of the infecting material there were several recoveries among those which received 0.001 to 0.002 to 0.003 gm., but all passed through a typical infection with all its symptoms. It is extremely doubtful, therefore, whether salvarsan or neosalvarsan mitigated the severity of the infection. The fact is noteworthy that in the same series of experiments the guinea pigs receiving 0.00005 and 0.0001 gm., or thereabout, of salvarsan died 1 to 2 days sooner than the controls, which died in 6 to 7 days. This suggests a possible earlier injury of the kidneys by the drugs, giving the leptospiras an easier and earlier access to, and localization in this organ. The inefficacy or dubious therapeutic value of salvarsan and neosalvarsan against the experimental *icteroides* infection of guinea pigs presents a close analogy to the observations already made by several investigators with *Leptospira icterohæmorrhagiæ*.

Several series of test-tube experiments were also made to determine the direct effect of salvarsan and neosalvarsan on *Leptospira icteroides* cultures. It was found, the injurious effect of alkalinity being eliminated, that the leptospiras remain motile for at least 1 hour in a concentration weaker than 1:10,000 of salvarsan or 1:1,000 of neosalvarsan. But they become gradually sluggish and succumb to the effect of the drugs at the end of 18 to 24 hours. The highest dilution which killed the leptospira in 18 hours was somewhere near 1:200,000.

When added to a culture medium, salvarsan and neosalvarsan both suppressed the growth of *icteroides* when their concentration in the

medium was 1:200,000. Hence these two drugs are highly poisonous for *Leptospira icteroides*.

The serums derived from rabbits which received 0.05 gm. of salvarsan or neosalvarsan per kilo of body weight 1 hour before bleeding proved to be very different from a normal rabbit serum in their behavior toward *Leptospira icteroides*. In the salvarsanized or neosalvarsanized serums the leptospiras remained active for at least 1 hour but appeared somewhat sluggish at the end of 18 hours, and were all dead and degenerated when examined after 48 hours. On the other hand, the leptospiras mixed with normal rabbit serum lived well and multiplied during the same period of time and under otherwise identical conditions (at 28°C.) To these tubes another portion of culture was added to determine whether or not a rapidly detrimental toxic substance had appeared in the drugged serum while standing for 72 hours, but the organisms remained still active at the end of 1 hour, 24 hours being required to kill them. In another experiment the salvarsanized and neosalvarsanized serums, together with normal serum as a control, were first left standing for 72 hours, after which period a rich culture of *icteroides* was introduced. The organisms remained uninfluenced for 1 hour in all the serums, but at the end of 24 hours many of those in the drugged serums were dead, and none was left alive at the end of 48 hours. In normal serum they steadily increased in numbers and were all active.

It is evident, then, that salvarsan or neosalvarsan introduced intravenously into the body of the rabbit is present in some form in the blood serum drawn at the end of 1 hour. The substance present in such serum has a slowly operating injurious effect upon *Leptospira icteroides*. The action of the drugs seems to be slower after passage through the animal body than before. If this phenomenon were to take place also in the infected body injected with these drugs, it is obvious that in a rapidly evolving infectious disease like yellow fever the progress of the infection will be too rapid to allow the drugs to exert their beneficial effect upon the course of the disease.

In direct contrast to the behavior of salvarsan and neosalvarsan *in vivo* and *in vitro*, anti-*icteroides* immune horse serum in a dose of 0.0001 cc., or 1 cc. of a 1:10,000 dilution, protected guinea pigs from an infection with at least 5,000 minimum lethal doses of *icteroides*

when injected simultaneously, but the same serum failed to exert any injurious effect upon the organism when mixed *in vitro* in a concentration weaker than 1:2,000. A rapid disintegration resulted with a concentration of 1:20 and almost complete agglutination and degeneration in 1:200.

The contrast between chemotherapy, as carried out with salvarsan and neosalvarsan, and serotherapy demonstrated with an immune serum is apparently of considerable practical significance.

STUDIES ON EXPERIMENTAL PNEUMONIA.

VIII. Experimental Streptococcus hæmolyticus Pneumonia in Monkeys.

By FRANCIS G. BLAKE, M.D., and RUSSELL L. CECIL, M.D.

(From the Bacteriological Laboratories of the Army Medical School, Washington.)

Plates 35 to 44.

(Received for publication, May 5, 1920.)

It has been shown in preceding papers[1,2] that pneumonia may be produced readily in normal monkeys by the intratracheal injection of pneumococcus and that the disease so produced is essentially identical in its clinical course, complications, and pathology with pneumococcus lobar pneumonia in man. In this paper experiments dealing with the production of pneumonia in monkeys with *Streptococcus hæmolyticus* are presented. These experiments were undertaken to determine whether hemolytic streptococcus pneumonia as it occurs in man could be reproduced experimentally in animals and for comparison with pneumococcus lobar pneumonia in monkeys. In addition the pathogenesis of hemolytic streptococcus pneumonia has been studied.

Method.

Monkeys were inoculated by direct intratracheal injection of broth cultures of *Streptococcus hæmolyticus*, and observations on the disease produced were made by the methods described in a preceding paper[1] on the production of pneumococcus pneumonia in monkeys. The strain of *Streptococcus hæmolyticus*[3] employed was originally isolated at autopsy from the lungs of a case of bronchopneumonia at Fort Sam Houston, Texas, in February, 1918, and possessed the typical

[1] Blake, F. G., and Cecil, R. L., *J. Exp. Med.*, 1920, xxxi, 403.

[2] Blake, F. G., and Cecil, R. L., *J. Exp. Med.*, 1920, xxxi, 445.

[3] We are indebted to Dr. A. R. Dochez for the strain of *Streptococcus hæmolyticus* used.

characteristics of this group of streptococci. It was a strongly Gram-positive coccus growing in chains of moderate length, bile-insoluble, and actively hemolytic. On the surface of rabbit blood agar plates it showed a wide zone of hemolysis, and when an 18 hour broth culture was mixed in equal quantity with a 5 per cent salt solution suspension of washed rabbit blood cells hemolysis was complete in 2 hours. It fermented lactose and salicin and failed to ferment mannitol, inulin, and raffinose. Its virulence had been raised by repeated mouse passage until 0.00000001 cc. of broth culture was lethal for a white mouse in 24 hours. Since this strain of *Streptococcus hæmolyticus* proved much less virulent for normal monkeys than the strains of pneumococcus employed in former experiments, the amount of culture injected varied from 0.1 to 10 cc. of an 18 hour plain broth culture. When larger amounts than 1 cc. were used the organisms were thrown down by centrifugalization and resuspended in portions of the supernatant broth so that the required amount of culture was contained in 1 cc. of medium, thus maintaining a constant amount of fluid injected into the trachea. Except in three instances, as indicated in the text, normal monkeys were used.

EXPERIMENTAL.

Production of Interstitial and Confluent Lobular Pneumonia by the Intratracheal Injection of Streptococcus hæmolyticus.

Nine normal monkeys were injected intratracheally with *Streptococcus hæmolyticus* with the successful production of varying degrees of interstitial or confluent lobular pneumonia in all the animals. The pneumonia was complicated in three cases by an extensive sero-fibrinous pleurisy, in one by pulmonary abscesses. It will be seen from Table I, in which the data of the experiments are summarized, that the disease was relatively mild. Death occurred in only three instances, in all of which a large amount of streptococcus was injected. Such a result is not surprising when it is recalled that *Streptococcus hæmolyticus* rarely attains sufficient virulence to produce a primary pneumonia in man, the pneumonia being nearly always secondary to measles, influenza, or some other preceding respiratory infection.

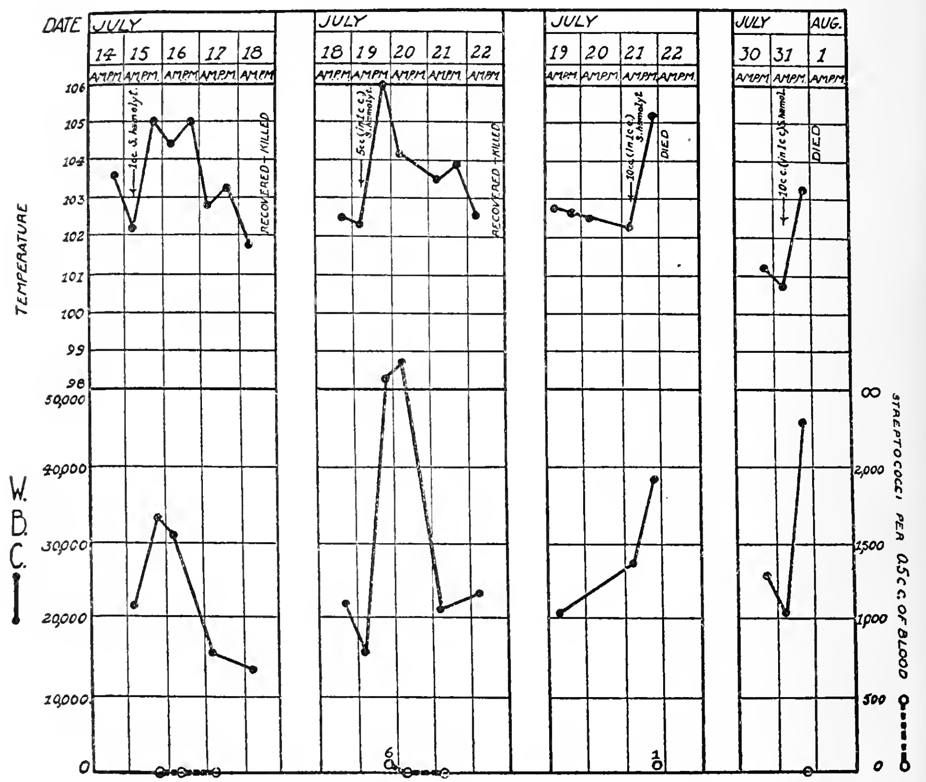
TABLE I.

Effect of Intratracheal Injection of Monkeys (Macacus syrichtus) with Streptococcus hæmolyticus.

Monkey No.	Weight.	Date of injection.	Amount of culture.	Result.	Autopsy.
	gm.	*1919*	*cc.*		
120	2,800	July 15	1.0	Pneumonia. Recovered on 4th day. Killed.	Interstitial pneumonia, L.L.; acute fibrinous pleuritis, left; chronic fibrous pleuritis, left.
121	3,150	" 15	1.0	Pneumonia. Recovered on 3rd day. Killed.	Interstitial and lobular pneumonia, R.L., R.M.
119	3,180	" 19	5.0 (in 1.0)	Pneumonia. Recovered on 4th day. Killed.	Interstitial and lobular pneumonia, R.L., R.U.
126	1,965	" 21	10.0 (" 1.0)	Pneumonia. Empyema. Died within 24 hrs.	Confluent lobular pneumonia, R.L., C.; engorgement and edema, R.M., R.U., L.L., L.M., L.U.; serofibrinous pleuritis, bilateral.
125	2,250	" 23	5.0 (" 1.0)	Pneumonia. Empyema. Died within 24 hrs.	Confluent lobular pneumonia, R.U., R.M., R.L., L.L.; engorgement and edema, L.M., L.U.; acute serofibrinous pleuritis, right; acute mediastinitis.
108	2,210	" 29	3.0 (" 1.0)	Pneumonia. Recovered on 4th day.	No autopsy.
123	2,445	" 29	5.0 (" 1.0)	Pneumonia. Killed on 2nd day.	Interstitial and lobular pneumonia, R.L.
128	2,650	" 31	10.0 (" 1.0)	Pneumonia. Empyema. Died within 24 hrs.	Confluent lobular pneumonia, R.U., R.L.; engorgement and edema, R.M.; acute serofibrinous pleuritis, right; chronic fibrous pleuritis, bilateral.
122	3,375	Aug. 6	0.1	Pneumonia; lung abscesses. Killed Aug. 14.	Interstitial pneumonia, R.L.; acute fibrinous pleuritis, right; pulmonary abscesses, R.L.

R.L., R.M., R.U., etc., indicate lobes of the lung.

The clinical features and pathology of hemolytic streptococcus pneumonia in monkeys are presented in the following protocols. The temperature, leucocyte counts, and blood cultures are shown in Text-fig. 1.



TEXT-FIG. 1, *a*, *b*, *c*, and *d*. Clinical course of experimental streptococcus pneumonia following the intratracheal injection of *Streptococcus hæmolyticus*. (*a*) Monkey 120; (*b*) Monkey 119; (*c*) Monkey 126; (*d*) Monkey 128.

Experiment 1.—Monkey 120 (Text-fig. 1, *a*). *Macacus syrichtus*, female; weight 2,800 gm. July 14, 1919. Well and active. July 15, 10.30 a.m. Intratracheal injection of 1 cc. of 18 hour broth culture of *Streptococcus hæmolyticus*. 4 p.m. Appears moderately sick; respirations accelerated. July 16. Moderately sick; respirations accelerated but without expiratory grunt; no cough. July 17.

Condition the same; no definite physical signs of consolidation. July 18. Much improved; breathing quietly. 10.30 a.m. Killed.

Autopsy. Anatomical Diagnosis.—Interstitial pneumonia, left lower lobe; acute fibrinous pleuritis, left; chronic fibrous pleuritis, left.

Pleural cavities.—Contain no fluid; there is fresh fibrin over the posterior surface of the left lower lobe; there are old fibrous adhesions between the visceral and parietal pleuræ on the left; right pleura appears normal. *Lungs.*—The posterior surface of the left lower lobe shows a mottled, deep red area spreading downward from the hilum; the cut surface yields a moderate amount of frothy fluid and presents minute deep red areas apparently of perivascular and peribronchial distribution; there is no firm consolidation; the other lobes appear normal.

Cultures.—Heart's blood and left lower lobe, no growth.

Macroscopic Examination.—Histological section through the left lower lobe (Fig. 1) shows considerable thickening of the vascular adventitia throughout the lobe. This process is particularly conspicuous about the pulmonary veins. There is a less marked thickening of the bronchial walls and interlobular septa in many places. In the upper and inner portion of the lobe there is a wedge-shaped area extending outward from the main bronchus to the pleura in which the alveolar structure appears much thickened, the alveoli atelectatic. There is little or no exudate in the alveoli.

Microscopic Examination.—*Left lower lobe.*—There is a thin deposit of fibrin on the pleural surface. The pleura is considerably thickened by the formation of new connective tissue, the interstices of which are infiltrated with many large mononuclear cells, plasma cells, lymphocytes, and a few polymorphonuclear leucocytes. The perivascular tissue is everywhere greatly thickened and infiltrated with large mononuclear cells, plasma cells, and lymphocytes, and occasional polymorphonuclear leucocytes (Fig. 2). In many places the interlobular septa and the walls of the bronchi are similarly thickened and infiltrated. The alveolar walls are conspicuously thickened and infiltrated with mononuclear cells and occasional polymorphonuclear leucocytes (Fig. 2), this process being especially prominent in the alveoli which lie adjacent to the blood vessels, bronchi, septa, and pleura. In places there is considerable proliferation of the epithelial cells lining the alveoli, an occasional mitotic figure being seen. The alveoli are for the most part air-containing and in the more extensively involved portions are atelectatic. A few scattered alveoli contain a contracted coagulum in which desquamated epithelial cells and a few leucocytes are embedded (Fig. 2), others contain a dense coagulum without cells, and still others contain a few red blood corpuscles. The bronchi contain little or no exudate, and the epithelium is intact. The epithelium of some of the terminal bronchioles has been desquamated, and the lumina contain a coagulum in which epithelial cells and a few leucocytes are embedded. The lymphatics of the larger bronchi and vessels near the hilum are filled with mononuclear cells. No streptococci are seen. *Bronchial lymph node.*—The marginal sinus is filled with large mononuclear cells and lymphocytes.

Monkey 120 represents a comparatively mild case of pneumonia with recovery on the 4th day. It exhibited no symptoms other than an ill defined appearance of being sick and a moderately accelerated respiratory rate. The clinical chart (Text-fig. 1, *a*) shows a prompt elevation of temperature 6 hours after inoculation with return to normal by rapid lysis, a leucocytosis, which was polymorphonuclear in character, during the acute stage of the disease, and sterile blood cultures throughout. Pathologic examination showed pneumonia in the left lower lobe, the most striking feature of which was its almost entirely interstitial character. The limitation of the process to the framework of the lung would appear to be an expression of a considerable degree of resistance to the infection, a supposition well supported by the brief duration of the disease and the promptness with which the lungs were freed from streptococci. There is no evidence that the disease began as a primary infection of the terminal bronchioles.

Experiment 2.—Monkey 119 (Text-fig. 1, *b*). *Macacus syrichtus*, female; weight 3,180 gm. July 18, 1919. Well and active. July 19, 10.15 a.m. Intratracheal injection of 5 cc. (in 1 cc.) of 18 hour broth culture of *Streptococcus hæmolyticus*. 4.15 p.m. Appears moderately sick; breathing rapidly. July 20. Quiet; respirations accelerated but without expiratory grunt; no cough; slight dullness with diminished breath sounds in right lower axilla; no râles. July 21. Moderately sick; respirations moderately accelerated. July 22. Appears better; apparently recovering; temperature has returned to normal by lysis. 3 p.m. Killed.

Autopsy. Anatomical Diagnosis.—Interstitial and lobular pneumonia, right upper and lower lobes.

Pleural cavities.—Contain no fluid. *Lungs.*—Over the posterior surface of the right lower lobe there is an extensive, pinkish gray area (Fig. 3), more translucent than the rest of the lung and sharply defined from the surrounding pale pink surface. On palpation this area is firm and is found to occupy a considerable part of the substance of the lobe. The cut surface shows an area of consolidation involving the posterior half of the lobe and sharply demarcated from the surrounding tissue. The surface of the consolidated area presents a pinkish gray, translucent appearance, and is slightly moist. There is a similar but smaller area in the right upper lobe. The remainder of the lungs is pale pink and crepitant. Mucosa of the trachea and bronchi appears normal.

Cultures.—Heart's blood and right lower lobe, no growth; right bronchus, *Streptococcus hæmolyticus.*

Microscopic Examination.—*Right lower lobe.*—The pleura is moderately thickened and infiltrated with large mononuclear cells, lymphocytes, and a few poly-

morphonuclear leucocytes. Young connective tissue cells are beginning to appear. In the pneumonic area the framework of the lung is conspicuously thickened and infiltrated with large mononuclear cells, lymphocytes, plasma cells, and polymorphonuclear leucocytes. This process is especially prominent in the vascular adventitia and is also quite marked in the walls of the bronchi and bronchioles (Fig. 4). The alveolar walls are thickened by swelling and active proliferation of the epithelium which, in places, shows a striking cuboidal appearance, and by moderate infiltration with mononuclear cells and leucocytes (Fig. 5). Nearly all the alveoli contain coagulated serum. In those lying adjacent to the bronchioles and blood vessels there are numerous desquamated epithelial cells, mononuclear phagocytic cells, and polymorphonuclear leucocytes (Fig. 4). In places this exudate may be traced into the lumen of a terminal bronchiole. There are several small areas in which there is considerable necrosis of the alveoli and the exudate is composed largely of polymorphonuclear leucocytes and granular debris. The smaller bronchi contain a moderate amount of exudate, and in places considerable desquamation of epithelium has occurred. No streptococci are seen. *Right upper lobe.*—Presents a similar picture. The process, however, is less extensive and more limited to the interstitial tissue.

Monkey 119 presented a clinical picture quite similar to that of Monkey 120, but appeared somewhat sicker. A temporary invasion of the blood stream by the streptococcus occurred. This probably should not be regarded as anything more than an overflow from the lungs following the inoculation of a large amount (5 cc.) of streptococcus. Autopsy showed a well defined area of interstitial and lobular pneumonia in the right lower lobe and a smaller area in the right upper lobe. Examination of histological sections showed a conspicuous involvement of the framework of the lung similar to that in Monkey 120. In addition, there were areas of lax consolidation, the location of which suggests that the infection had spread outward from the peribronchial and perivascular tissue to the adjacent alveolar structure. The preponderance of mononuclear cell infiltration, the presence of fibroblasts, the active proliferation of alveolar epithelium, and the absence of streptococci indicate that the infection had terminated and that the process of repair had begun.

Experiment 3.—Monkey 126 (Text-fig. 1, *c*). *Macacus syrichtus*, female; weight 1,965 gm. July 19 and 20, 1919. Well and active. July 21, 11 a.m. Intratracheal injection of 10 cc. (in 1 cc.) of 18 hour broth culture of *Streptococcus hæmolyticus*. 4.15 p.m. Appears moderately sick; breathing very rapidly. 11 p.m. Appears very sick; breathing difficult and very rapid. July 22. Died during night.

Autopsy. Anatomical Diagnosis.—Confluent lobular pneumonia, right lower and cardiac lobes; engorgement and edema of right middle and upper, and left lower, middle, and upper lobes; serofibrinous pleuritis, bilateral.

Right pleural cavity.—Contains a considerable quantity of slightly turbid, claret-colored fluid. *Left pleural cavity.*—Contains a smaller amount of similar fluid. *Lungs.*—Voluminous and intensely congested, the surfaces of both lungs being deep pink and thickly mottled with large, dark red, irregular patches. The right lower lobe is fairly firm and nodular on palpation. The cut surface is dark red and oozes a large quantity of claret-colored fluid. On scraping the surface scattered areas of grayish red consolidation are seen. These are elevated and in many cases confluent. The cardiac lobe shows an almost uniform lax consolidation. The cut surface is dark red, moist, and homogeneous. The right middle and upper lobes show intense congestion, the cut surfaces yielding a considerable amount of frothy, blood-tinged fluid, but no definite consolidation is made out. The left lower, middle, and upper lobes present a similar picture. Trachea and bronchi are filled with a bloody, mucoid exudate, but the mucosa appears normal.

Cultures.—Heart's blood, right lower lobe, and right pleural fluid, *Streptococcus hæmolyticus*.

Macroscopic Examination.—Histological section through the right lower lobe shows an intense edema of the vascular adventitia and bronchial walls which are distended to many times their normal thickness. This is most marked about the larger bronchi and vessels. There is intense vascular engorgement. The alveoli lying adjacent to the larger bronchial branches and to the vessels are filled with serous exudate, while those lying midway between and at the periphery are to a considerable extent air-containing. There are several small irregular areas adjacent to some of the larger veins and bronchi where consolidation is present.

Microscopic Examination.—*Right lower lobe.*—There is a layer of fibrin infiltrated with polymorphonuclear leucocytes on the pleural surface. The pleura is moderately thickened, infiltrated with leucocytes, and in places appears necrotic. The lymphatics of the pleura are greatly distended. In many places they are thrombosed and contain dense masses of streptococci. The bronchial walls and vascular adventitia are greatly distended with edema and infiltrated to a variable degree with polymorphonuclear leucocytes. Dense masses of streptococci are seen in the outer margins of the perivascular tissue. They are also present, though less numerous, in the bronchial walls. The lymphatics of the vessels and bronchi are distended, and are frequently filled with thrombi, leucocytes, or masses of streptococci. The interlobular septa are edematous. The bronchi show epithelial desquamation and in places necrosis of the walls. Many of them contain leucocytes, coagulated serum, and necrotic debris. Streptococci are present in this exudate. The walls of the terminal bronchioles are thickened and infiltrated with leucocytes and streptococci. The epithelium is desquamated. Those in the peripheral portions of the lobe contain no exudate, while many of those in the more central portions where consolidation has occurred

contain leucocytes and necrotic material. In the central portions of the lobe and extending outward along the main bronchial and vascular branches the alveoli are filled with exudate. Those lying adjacent to the bronchi and vessels contain great numbers of polymorphonuclear leucocytes embedded in coagulated serum and some fibrin, those more distant only coagulated serum. The alveolar walls are thin and show no cellular infiltration. In many places there is extensive necrosis of the exudate and alveolar walls. Immense numbers of streptococci are present in the exudate, being particularly numerous in the necrotic areas. In the peripheral portions of the lobe many of the alveoli are air-containing, while others lying adjacent to the vessels, pleura, and bronchioles contain coagulated serum. The walls of the alveoli and atria are edematous, and in places about the terminal bronchioles, alveolar ducts, and vessels are infiltrated with polymorphonuclear leucocytes (Fig. 6). The distribution of streptococci in this portion of the lobe is particularly interesting. Where exudation into the alveoli and bronchioles has not occurred, streptococci are seen in the walls of the terminal bronchioles and in the walls of the adjacent alveoli where they lie between the capillary and the lining epithelium (Fig. 7), but none are seen in the alveolar spaces or in the lumina of the bronchioles. Where serous exudate has poured out into the alveoli streptococci are present in large numbers in the exudate. *Right upper lobe.*—The process is much less advanced and there is no consolidation. The vessels and capillaries are greatly engorged. There is moderate edema of the vascular adventitia and bronchial walls but no cellular infiltration. The interlobular septa are not prominent. There is some desquamation of the bronchial epithelium but the bronchi and bronchioles contain no exudate. The alveoli are air-containing except in a few scattered patches adjacent to the bronchi where they contain a serous exudate. About some of the bronchi and terminal bronchioles the alveolar walls appear moderately edematous. The perivascular and peribronchial lymphatics are distended but contain few cells. Many streptococci are seen on the epithelial surface of a large bronchus and may be seen penetrating the subepithelial layers where they are present in large numbers. In the smaller bronchi and terminal bronchioles of the central portions of the lobe they are present in large numbers in the walls (Fig. 8), but not on the epithelial surface or in the lumen. None are seen in the walls of the peripheral bronchioles. Large numbers are present in the walls of the alveoli that lie adjacent to the bronchi, while the walls of the more distant alveoli are free from organisms. In a similar manner many streptococci are present in the walls of the alveoli in which terminate bronchioles whose walls have been invaded, while the walls of the alveoli in which unaffected bronchioles terminate are free from organisms. Where a serous exudate has poured out into the alveoli streptococci are present in the exudate. In places streptococci are seen in the perivascular lymphatics. At a few points they have reached the pleura but have not spread out in dense masses through the pleural lymphatics.

Experiment 4.—Monkey 125. *Macacus syrichtus*, male; weight 2,250 gm. July 22, 1919. Well and active. July 23, 11 a.m. Intratracheal injection of 5

cc. (in 1 cc.) of 18 hour broth culture of *Streptococcus hæmolyticus*. 4.15 p.m. Quiet; breathing very rapidly; moderate dullness and suppressed breath sounds over right lower lobe. Blood culture shows 1 colony of *Streptococcus hæmolyticus* per 0.5 cc. of blood. 11 p.m. Very sick; respirations difficult and very rapid; evidently suffering from severe pleural pain; crying frequently. July 24. Died between midnight and 9 a.m.

Autopsy. Anatomical Diagnosis.—Confluent lobular pneumonia, right lower, middle, and upper lobes, left lower lobe; engorgement and edema, left middle and upper lobes; acute serofibrinous pleuritis, right; acute mediastinitis.

Anterior mediastinum.—Shows intense congestion and edema of the tissues. *Right pleural cavity.*—Filled with slightly turbid, claret-colored fluid, the right lung being partially collapsed. *Left pleural cavity.*—Contains no fluid. *Lungs.*—The surface of the entire right lung is intensely congested and covered with dark red, blotchy patches, most marked in the middle and lower lobes. The whole lung feels heavy and soggy, with irregular patches of moderately firm consolidation, most marked in the lower lobe. The cut surface of the lower lobe is dark red and shows irregular, patchy, consolidated areas, which become confluent in some places. It yields a large quantity of bloody fluid. The middle and upper lobes present a similar picture, but the consolidation is less marked. The left lung is voluminous, the anterior surfaces being pale pink. Posteriorly the surfaces show irregular patches of a deep red color. On palpation there is an area of moderately firm, nodular consolidation in the upper and posterior portion of the lower lobe. The cut surface yields a considerable amount of bloody fluid and shows a well demarcated area of confluent lobular consolidation in the upper and posterior portion, the surrounding air-containing tissue being intensely congested and edematous. The cut surfaces of the middle and upper lobes are congested and yield a moderate amount of frothy, blood-tinged fluid. Trachea and bronchi are congested and contain a bloody, mucoid exudate.

Cultures.—Heart's blood, right lower lobe, left lower lobe, pleural fluid, and bronchi, *Streptococcus hæmolyticus*.

Macroscopic Examination.—Histological section through the right lower lobe (Fig. 9) shows intense vascular engorgement. There is marked perivascular edema which is particularly conspicuous about the larger vessels. The bronchial walls appear moderately thickened. In the lower half of the lobe there is a fairly large, roughly wedge-shaped area of consolidation extending downward toward the periphery. This area of consolidation merges indefinitely into a surrounding zone of edema which in turn merges into a peripheral zone of alveoli which are largely air-containing. In the upper portion of the lobe there is a similar, though smaller area of consolidation with ill defined margins merging into a surrounding zone of edema. Histological section through the left lower lobe presents a similar picture. Areas of consolidation appear as roughly oval or circular areas surrounding a central vein or bronchus. The margins of the consolidated areas are irregular and merge without sharp demarcation into surrounding zones of edema. The outer and lower portions of the lobe show no consolidation, the alveoli being largely air-containing.

Microscopic Examination.—Right lower lobe.—There is a deposit of fibrin on the pleura. The pleura is edematous and infiltrated with a moderate number of leucocytes and dense masses of streptococci. The lymphatics are distended and contain masses of streptococci, leucocytes, and large mononuclear cells. In many places they are thrombosed. The perivascular tissue is greatly distended with edema, particularly about the pulmonary arteries, but shows very little cellular infiltration (Fig. 10). The interlobular septa are also very edematous. Dense masses of streptococci are seen in the lymphatics and outer layers of the tissue surrounding the pulmonary veins (Fig. 11). The vessels and capillaries are greatly engorged and in many places the latter show thrombi. The bronchial walls are moderately thickened and infiltrated with polymorphonuclear leucocytes and mononuclear cells, and their lymphatics are distended. In places the walls are necrotic and have completely lost their structure. There is extensive desquamation of the epithelium of the bronchi and bronchioles. The larger bronchi and the bronchioles of the central portions of the lobe contain leucocytes and necrotic debris in which many streptococci are seen. The terminal bronchioles of the peripheral portions show desquamation of epithelium, thickening and infiltration of their walls with polymorphonuclear leucocytes and streptococci, but contain no exudate or streptococci. In the central portions of the lobe, particularly in relation to the bronchi and vessels, the alveoli are filled with an exudate of polymorphonuclear leucocytes and small numbers of mononuclear cells embedded in coagulated serum and fibrin (Fig. 10). In places there is also hemorrhage into the alveoli. The alveolar walls are not distended. Streptococci in pairs and short chains are everywhere seen through this exudate. In many areas extensive necrosis with almost complete destruction of the alveolar walls has occurred. Dense masses of streptococci are present in these necrotic areas. Surrounding the areas of consolidation the alveoli show extensive edema and some hemorrhage. In the peripheral portions of the lobe, except immediately adjacent to the pleura, the alveoli are largely air-containing. About the terminal bronchioles and adjacent to the veins their walls are distended with edema and show some cellular infiltration. Streptococci are numerous in the walls of these comparatively intact alveoli, but are not seen in the alveolar spaces. *Cardiac, right middle, and right upper lobes.*—Sections show the same picture though the process is less widespread. A section from the central portion of the right upper lobe shows a point of local injury to the mucosa of a large bronchus (Fig. 12). The epithelium is desquamated, the subepithelial tissue is infiltrated with polymorphonuclear leucocytes, and numerous streptococci are seen invading the bronchial wall (Fig. 13).

Experiment 5.—Monkey 128 (Text-fig. 1, *d*). *Macacus syrichtus*, female; weight 2,650 gm. July 30, 1919. Well and active. July 31, 11.15 a.m. Intratracheal injection of 10 cc. (in 1 cc.) of 18 hour broth culture of *Streptococcus hæmolyticus*. 4.30 p.m. Appears moderately sick; breathing very rapidly. Aug. 1. Died during night.

Autopsy. Anatomical Diagnosis.—Confluent lobular pneumonia, right lower and upper lobes; engorgement and edema, right middle lobe; acute serofibrinous pleuritis, right; chronic fibrous pleuritis, bilateral.

Right pleural cavity.—Contains a moderate amount of slightly turbid, claret-colored fluid. *Left pleural cavity.*—Contains no fluid. There are scattered old fibrous adhesions in both pleural cavities. *Lungs.*—The right lung is covered with old fibrous tags and the lobes are bound together by old fibrous adhesions. The surface of the upper lobe is congested and marked at many points by small dark red blotches. The cut surface appears congested and yields a considerable amount of blood-tinged fluid. There is some peribronchial consolidation. The middle and lower lobes are intensely congested and feel heavy and soggy. On palpation the lower lobe shows a moderately firm, nodular area of consolidation in the lower portion. The cut surfaces of the middle and lower lobes are dark red and ooze a large quantity of bloody fluid. In the lower lobe there is an irregular patch of consolidation in the lower portion, dark red in color, and somewhat elevated above the surrounding edematous and air-containing tissue. The left lung appears moderately congested but is everywhere air-containing and free from consolidation.

Cultures.—Heart's blood, pleural fluid, and right lower lobe, *Streptococcus hæmolyticus.*

Microscopic Examination.—*Right lower lobe.*—The pleura is greatly thickened by old fibrous tissue, the interstices of which are infiltrated with polymorphonuclear leucocytes, mononuclear cells, and moderate numbers of streptococci. The pleural lymphatics are greatly distended and everywhere contain dense masses of streptococci. The vessels are greatly engorged and many of the smaller vessels and capillaries contain thrombi. The perivascular tissue is edematous and infiltrated with leucocytes. Dense masses of streptococci are present in the distended lymphatics and outer layers of the adventitia of the pulmonary veins (Fig. 14). The bronchial walls are thickened and infiltrated with leucocytes and streptococci and in places are completely necrotic. The epithelium is desquamated. The walls of the terminal bronchioles are similarly thickened and infiltrated. The interlobular septa are edematous, infiltrated with polymorphonuclear leucocytes, and their lymphatics are distended and filled with streptococci, leucocytes, and thrombi. Except at the peripheral portions the alveoli are edematous and contain many disintegrating leucocytes. Much of the tissue is undergoing necrosis, the structure of the alveolar walls being indistinct (Fig. 14). Streptococci are abundant in these areas. At the periphery the involvement is largely interstitial without exudation into the alveoli. The alveolar walls are greatly distended with edema, and infiltrated with leucocytes, and contain abundant streptococci which, however, are not seen in the alveolar spaces.

Monkeys 126, 125, and 128 illustrate very severe and rapidly fatal cases of streptococcus pneumonia associated with the early develop-

ment of serofibrinous pleurisy. The extremely rapid respiratory rate exhibited by these monkeys was undoubtedly due to the rapid accumulation of a large pleural exudate. The almost negligible invasion of the blood stream by the streptococcus 6 hours after inoculation, in spite of the overwhelming nature of the infection and the large numbers of streptococci injected, is striking. Monkeys receiving much smaller intratracheal injections of pneumococcus invariably showed a very heavy septicemia 6 hours after inoculation. It is noteworthy that none of these monkeys, though very severely infected, showed any evidence of prostration during the onset of the pneumonia, exhibiting no definite symptoms other than the rapid respiratory rate and an ill defined appearance of being sick, yet all died within 24 hours after inoculation.

The pathologic picture was one of rapid and widespread invasion of the lungs with little show of resistance on the part of the tissue. Proliferation of the alveolar epithelium, extensive mononuclear cell reaction, and limitation of the process to the interstitial tissue were lacking. The primarily interstitial nature of the infection, though somewhat masked by the widespread confluent areas of alveolar edema, consolidation, and necrosis, was, nevertheless, shown by the intense perivascular edema and leucocytic infiltration, the thickening and infiltration of the walls of the bronchi and bronchioles, and the edema of the interlobular septa. It was even more strikingly shown in the peripheral areas of the lung where the process was entirely interstitial in character without exudation into the alveolar spaces and lumina of the bronchioles. Since these were all cases of less than 24 hours duration the distribution of streptococci is of particular significance in indicating the mode of invasion of the lung. In the earliest lesion observed (Monkey 126, right upper lobe) streptococci are present on the epithelial surface of a large central bronchus, and where the epithelium is injured they may be seen penetrating into the peribronchial tissue. This is also well shown in a section from the right upper lobe of Monkey 125 (Fig. 13). In the smaller bronchi and bronchioles, however, they are seen only in the walls, and none are present in the lumina or on the epithelial surfaces. They are also present in the walls of alveoli that lie immediately adjacent to the bronchi and bronchioles whose walls are infected, but are not seen

elsewhere in the alveolar structure. A few are seen in the perivascular lymphatics, and in a few localized spots they have reached the pleura where they lie in masses in the pleural lymphatics. In the more advanced lesions they are present, in addition to the areas described above, in dense masses in the lymphatics about the pulmonary veins, in the interlobular septa, and in the pleura. Wherever exudate has poured out into the alveoli abundant streptococci are present in the alveoli, being particularly numerous in areas that are undergoing necrosis. But where exudation into the alveoli has not occurred the streptococci are confined within the alveolar walls, lying between the central capillary and the epithelial lining of the air space.

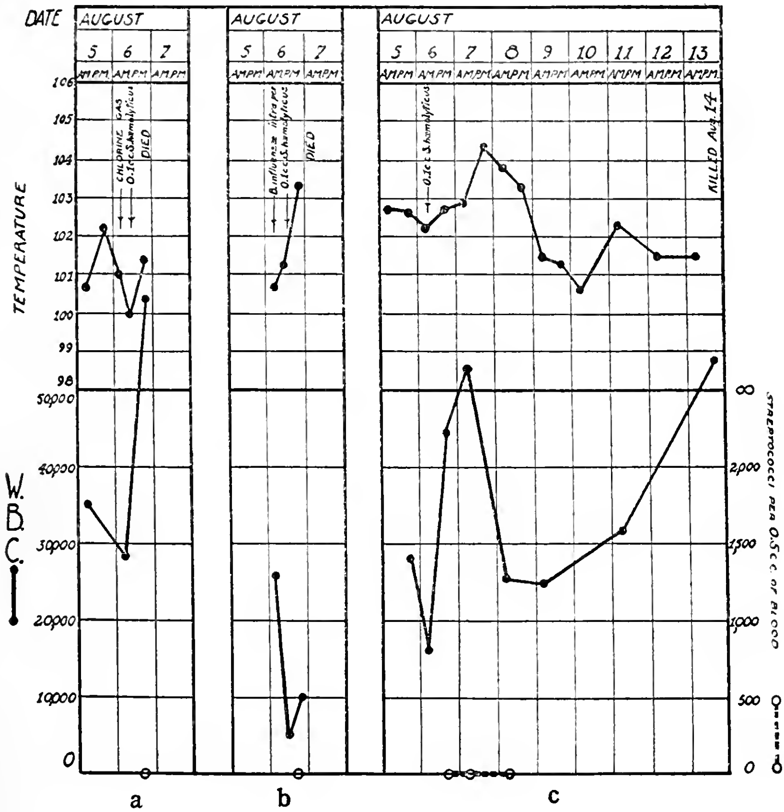
Effect of Preceding Injury to the Respiratory Tract and of Lowering of General Resistance on the Course of Experimental Streptococcus Pneumonia.

The preceding experiments have shown that the normal monkey is comparatively resistant to intratracheal injections of a highly virulent hemolytic streptococcus and succumbs only when large doses are employed. As has been pointed out, such a result is not out of harmony with the infrequent occurrence of primary streptococcus pneumonia in man, in whom the disease commonly occurs only as a secondary infection following some preceding disease affecting the respiratory tract.

It seemed of interest, therefore, to determine the effect of a preceding local injury to the respiratory tract and of the lowering of general resistance without local injury to the respiratory tract on the course of streptococcus pneumonia subsequently produced by the intratracheal injection of streptococci.

Experiment 6.—Four monkeys were used. Preliminary injury to the respiratory tract was produced in Monkey 19 by a few seconds exposure to chlorine gas 1 hour before intratracheal injection of *Streptococcus hæmolyticus*, in Monkey 129 by the intratracheal injection of 0.000001 cc. of Pneumococcus Type I 24 hours before intratracheal injection of streptococcus. Lowering of general resistance without injury to the respiratory tract was produced in Monkey 48 by the intraperitoneal injection of a strain of *B. influenzæ* possessing pathogenicity for monkeys 3½ hours before intratracheal injection of streptococcus. A normal monkey, No. 122, served as a control, receiving only an intratracheal injection of

streptococcus. A small amount of streptococcus, 0.1 cc., was injected in all the animals. The essential data of the experiment are shown in Table II and Text-figs. 2 and 3.



TEXT-FIG. 2, *a*, *b*, and *c*. Effect of preceding inhalation of chlorine gas (Monkey 19) and of intraperitoneal injection of *B. influenzæ* (Monkey 48) on the course of experimental streptococcus pneumonia produced by intratracheal injection of 0.1 cc. of broth culture of *Streptococcus hæmolyticus*. (*a*) Monkey 19; (*b*) Monkey 48; (*c*) Monkey 122, control.

The foregoing experiment shows that in the presence of a local injury to the respiratory tract caused by gassing with chlorine a small amount of *Streptococcus hæmolyticus*, which in a normal control monkey produced only a mild pneumonia localized in one lobe, produced a rapid and widespread infection of the lungs. It is, of course,

TABLE II.

Effect of Preceding Injury to the Respiratory Tract and of Lowering of General Resistance on the Course of Hemolytic Streptococcus Pneumonia.

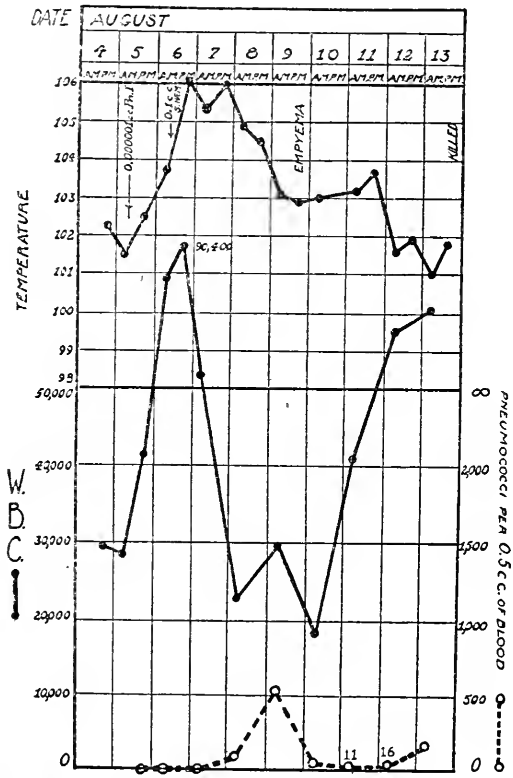
Monkey No.	Weight.	Preliminary procedures.	Condition at time of intratracheal injection of *S. hæmolyticus*.	Time of intratracheal injection of 0.1 cc. of *S. hæmolyticus*.	Result.	Autopsy.	
						Macroscopic pathology.	Cultures
	gm.	*1919*		*1919*			
19	4,000	Aug. 6, 10 a.m. Exposed for a few seconds to dilute chlorine gas.	Coughing, but otherwise appears well.	Aug. 6, 11 a.m.	Rapidly progressive pneumonia. Aug. 6, 5.30 p.m. Died.	Widespread confluent lobular pneumonia, all lobes, stage of engorgement and edema.	H.B., n.g. Br., S.H. Lungs, S.H.
48	4,785	Aug. 6, 10.15 a.m. Intraperitoneal injection of 13 cc. of salt solution suspension of *B. influenzæ*.	Lying prostrate; abdomen tense and tender; marked leucopenia.	Aug. 6, 1.50 p.m.	Rapidly progressive pneumonia. Aug. 7. Died.	Widespread confluent lobular pneumonia, R.L., R.M., R.U., L.M.; acute serofibrinous pleuritis, right; acute general peritonitis.	H.B., S.H., B.I. R.L., " Pl., " Tr., " Per., B.I., S.H.
129	2,530	Aug. 5, 11.15 a.m. Intratracheal injection of 0.000001 cc. of Pn. I.	Has developed lobar pneumonia; moderately sick.	Aug. 6, 10.35 a.m.	Lobar pneumonia. Developed empyema on 5th day. Aug. 13. Killed.	Lobar pneumonia, R.L., beginning resolution and organization; empyema, right.	H.B., Pn.I. Tr., " I. R.L., " I. Emp., " I.

122	3,375	None (control).	Well and active.	Aug. 6, 10.45 a.m.	Mild pneumonia with recovery by lysis on 4th day. Aug. 14. Killed.	Interstitial pneumonia, R.L.; acute fibrinous pleuritis, right; pulmonary abscesses, R.L.	H.B., n.g. Abscess, S.H.

R.L., R.M., R.U., etc., indicate lobes of the lung.

H.B. indicates heart's blood; Br., bronchus; Pl., pleural fluid; Tr., trachea; Per., peritoneum; n.g., no growth in cultures; S.H., *Streptococcus hæmolyticus*; B.I., *Bacillus influenzæ*; Pn. I, Pneumococcus Type I.

clear that chlorine poisoning was in large part the cause of the rapidly fatal outcome in Monkey 19, and it is not intended to imply that death was due to streptococcus pneumonia. It seems quite clear, nevertheless, that the pulmonary injury produced by chlorine greatly facilitated invasion of the lungs by the streptococcus.



TEXT-FIG. 3. Monkey 129. Effect of the intratracheal injection of 0.1 cc. of broth culture of *Streptococcus hæmolyticus* during the course of experimental Pneumococcus Type I pneumonia.

In a similar manner a widespread streptococcus pneumonia with pleural exudate developed in Monkey 48 which had previously received an intraperitoneal injection of *Bacillus influenzæ* sufficiently large to cause extreme prostration and marked leucopenia at the time streptococcus was injected into the trachea. Since there was no

preceding local injury to the respiratory tract in this case, other factors must have contributed to lack of resistance against streptococcus invasion of the lungs. It would seem probable that the destruction of leucocytic defense as evidenced by the marked leucopenia and by the almost entire absence of polymorphonuclear leucocytes in the pulmonary exudate (Fig. 15) played a considerable part. Whether other factors were concerned it is impossible to say.

Regarding Monkey 129 there is no certain evidence that the intratracheal injection of streptococcus in the presence of a pneumococcus pneumonia exerted any effect on the course of the disease. The early development of empyema during the course of the pneumococcus pneumonia is unusual, and suggests that the empyema may have been initiated by *Streptococcus hæmolyticus*. Failure to find streptococci in the empyema fluid at autopsy, however, throws considerable doubt on this assumption. Likewise, though study of histological sections from the lung suggested that the streptococcus was in part responsible for the lesion, there is no bacteriological evidence in support of this view, and the matter must remain in doubt.

The control monkey, No. 122, which had only a mild pneumonia largely of the interstitial type (Figs. 16 to 19) showed an important feature in the development of pulmonary abscesses (Fig. 16) in which innumerable streptococci were present.

Effect of Inoculating a Monkey in the Nose and Throat with Streptococcus hæmolyticus.

It has been shown[1] that normal monkeys may receive a large inoculation of virulent Pneumococcus Type I in the nose and throat without suffering any apparent ill effect. It seemed desirable, therefore, to determine the effect of *Streptococcus hæmolyticus* in this respect.

Experiment 7.—Monkey 135. *Macacus syrichtus*, male; weight 2,150 gm. Aug. 19, 1919. Well and active. 9.20 p.m. 2.5 cc. of 10 hour broth culture of *Streptococcus hæmolyticus* sprayed into nose and throat. Aug. 20 to 31. Remained well and active with no evidence of infection. Temperature and leucocyte count remained normal.

The above experiment shows that a virulent hemolytic streptococcus inoculated into the nose and throat of a normal monkey failed

to produce pneumonia. Furthermore, like the pneumococcus, it failed to initiate an infection of the upper respiratory tract or exert any apparent effect whatsoever.

DISCUSSION.

The foregoing experiments have shown that pneumonia produced in monkeys by the intratracheal injection of *Streptococcus hæmolyticus* differs in many respects, both clinically and pathologically, from pneumonia produced by the pneumococcus.[1,2] The differences exhibited appear to be quite similar to those which occur in man between hemolytic streptococcus pneumonia and pneumococcus lobar pneumonia.

Clinically, except in the animals which developed empyema, the disease was comparatively mild and was characterized by an inconstant type of temperature curve of 3 to 5 days duration with recovery by lysis. The respirations, though accelerated, were not characterized by the expiratory grunt commonly seen in lobar pneumonia in monkeys. Failure of the streptococcus to invade the blood stream except as a terminal event in the fatal cases was in striking contrast with the constant and early invasion of the blood by the pneumococcus. In all the above features the disease appears to be similar to streptococcus pneumonia in man. It was even more strikingly so in its complications and pathology. In four out of eleven cases (36.3 per cent) the early development of an abundant serofibrinous pleural exudate teeming with streptococci took place. In contrast with this, empyema developed but once (1.6 per cent) in 61 monkeys with experimental pneumococcus lobar pneumonia, and then only late in the disease. One monkey developed mediastinitis, one pulmonary abscesses, both of which conditions occur as complications of streptococcus pneumonia in man.

Particular interest attaches to the pathology of hemolytic streptococcus pneumonia because of the many detailed studies of the disease that have been made during the epidemics of streptococcus pneumonia of 1917 and 1918.

The views of MacCallum,[4] who has made an extensive study of the subject, may be summarized briefly. Two main types of pulmonary lesions are found, interstitial bronchopneumonia and lobular pneumonia. These two types may be, and frequently are combined in the same case. In both instances the infection is a descending one from the upper respiratory tract. In the interstitial type the pneumonia begins as a primary infection of the bronchioles which leads to great changes in their walls and in the adjacent pulmonary tissue with the formation of firm nodules of consolidation about the terminal portions of the bronchi. In the course of the affection thickening and infiltration of the bronchial and alveolar walls, of the interlobular septa, of the perivascular adventitial tissue, and of the pleura occur. The lymphatics of these structures are widely infected and thrombosed. The process is limited in large part to the framework of the lung. The picture of rapid organization and mononuclear cell reaction indicates the tendency of the lung to heal by barricading the further invasion of the infecting organisms. Streptococci are found in large numbers in the bronchial exudate, in the thrombosed lymphatics, and in the pleura, but few are present in the lung substance.

The second type, or lobular pneumonia, is composed of cases in which the streptococci invade in the same way. Little resistance is offered to the infection, however, and large patches of confluent lobular consolidation occur in which the alveoli are filled with blood, leucocytes, and enormous numbers of streptococci. The interstitial and mononuclear cell reaction, which are expressions of resistance, are wanting, and widespread areas of necrosis may occur. There are many cases in which this lobular pneumonia occurs in the pure form, but there are many others in which it is found side by side with the interstitial bronchopneumonia. They are different expressions of reaction to the same infection, the interstitial type being an expression of marked resistance, the lobular type one of feeble resistance.

It seems quite clear that the pneumonia produced in monkeys by *Streptococcus hæmolyticus* is essentially identical in its pathology with that which occurs in man. Furthermore, MacCallum's suggestion that the occurrence of interstitial pneumonia or lobular pneumonia or combinations of the two types depends upon the resistance offered to the infection would seem to be well supported by the results of our experiments, since with increasing doses of streptococci the pathologic picture passed from that of a purely interstitial pneumonia to that of a mixed interstitial and lobular pneumonia, and finally with very

[4] MacCallum, W. G., The pathology of the pneumonia in the United States Army camps during the winter of 1917–18, Monograph of The Rockefeller Institute for Medical Research, No. 10, New York, 1919.

large doses to that of a widespread confluent lobular pneumonia with necrosis.

There is, however, one important difference between the picture presented in monkeys and that described by MacCallum in man; namely, the site of the initial lesion and the primary mode of invasion of the lung by the streptococcus. MacCallum states that the initial lesion is one of the terminal bronchioles and that in the course of the affection thickening and infiltration of the bronchial and alveolar walls, of the interlobular septa, of the perivascular adventitial tissue, and of the pleura occur, the streptococcus invading by way of the lymphatics of these structures. In the disease in monkeys, on the other hand, the initial lesion, or point of invasion of the streptococcus, appears to be at one or more points in the walls of the larger or medium sized bronchi, with extension of the infection from these points through the lung by way of the lymphatics of the bronchial walls to the bronchioles and of the perivascular tissue and interlobular septa to the pleura. Study of early lesions would seem to indicate that infection of the alveoli is primarily by invasion of the alveolar walls by streptococci which spread out during the progress of the infection of the framework of the lung to the alveolar walls that lie adjacent to these structures. Injury of the walls and desquamation of the epithelium of the bronchioles and smaller bronchi with exudation of leucocytes into the lumen would appear to be secondary to invasion of the walls of the bronchioles and smaller bronchi by streptococci which have reached these points by way of the peribronchial lymphatics rather than by way of the bronchial lumen. In a similar manner exudation into the alveoli follows interstitial invasion of the alveolar walls and consequent injury to them. The constant tendency of the tissue to barricade the progress of the infection brings about the characteristic limitation of the alveolar lesions to the alveoli which lie adjacent to the bronchial and vascular trees, the septa, and the pleura, and only when this resistance gives way does a rapidly spreading confluent lobular pneumonia develop. It is, of course, possible that during the progress of the invasion of the peribronchial lymphatics no great injury to the surrounding alveolar tissue may be produced until the streptococci attain the lymphatics of the bronchioles, in which case the disease might appear as a primary infection of the bronchioles

as described by MacCallum. On the other hand, were it actually a primary infection of the bronchioles, it is difficult to correlate this conception with the very early and widespread infection of the perivascular tissue, interlobular septa, and pleura, which occurs even in areas where the terminal bronchioles and their adjacent alveoli remain unaffected. In this connection it should be remembered that the primary mode of invasion and the earliest lesions which may be easily studied in experimental animals, can rarely be studied in man, but must be inferred to a considerable extent from the picture found at later stages of the disease, and that in man the pathology of streptococcus pneumonia is frequently complicated by a preceding and often concomitant bronchial infection with other organisms.

It will be seen from the foregoing discussion that the mode of invasion of the lung by *Streptococcus hæmolyticus* is essentially identical with the mode of invasion by the pneumococcus as described in a preceding paper on the pathogenesis of lobar pneumonia.[2] It is furthermore apparent that the first response of the animal to this invasion is the same in both cases; namely, vascular engorgement and interstitial edema with polymorphonuclear leucocytic infiltration. Following the stage of lymphatic and interstitial invasion, however, the end-results are divergent. In pneumococcus lobar pneumonia little resistance is offered by the tissue to the invading organisms and the process sweeps rapidly through the lobe, ultimately producing a comparatively homogeneous complete lobar consolidation without necrosis or extensive damage to the bronchi. In streptococcus interstitial pneumonia on the other hand, an almost immediate resistance to the invading organisms is offered, the process is limited in large part to the interstitial tissue and lymphatics of the pulmonary framework and to the alveoli immediately adjacent, and the picture of active repair is encountered. If, on the other hand, comparatively little resistance is offered to the infection, the process sweeps through the lobe, producing a confluent lobular pneumonia which ultimately may lead to consolidation of an entire lobe. In this respect the disease is analogous to lobar pneumonia. There is, however, a striking difference between the two which would appear to depend upon the power of the streptococcus to produce marked local injury to the tissues, resulting in extensive damage to the walls of the bronchi

and in widespread areas of alveolar necrosis, features which are not often seen in pneumococcus pneumonia. The greater tendency for extensive organization to occur following streptococcus pneumonia is another feature in which the two diseases differ. It would seem possible that this difference may be dependent upon the more extensive tissue injury caused by the streptococcus than by the pneumococcus.

SUMMARY.

1. Pneumonia has been consistently produced in normal monkeys by intratracheal injection of *Streptococcus hæmolyticus.*

2. The pneumonia produced has been shown to be comparable with hemolytic streptococcus pneumonia in man with respect to its clinical features, complications, and pathology.

3. Two pathologic types of the disease have occurred, interstitial pneumonia and confluent lobular pneumonia. Both types have been found in the same animal.

4. The type of pneumonia has appeared to be dependent upon the amount of streptococcus culture injected, interstitial pneumonia following the injection of small amounts and being an expression of considerable resistance, confluent lobular pneumonia following the injection of large amounts and being an expression of comparative lack of resistance.

5. Study of the distribution of streptococci in the lungs and of the character of the lesions in early stages of the disease has shown that streptococci may primarily invade the pulmonary tissue by penetration of the walls of the larger bronchial branches and that they are distributed from the points of invasion by way of the peribronchial, perivascular, and septal interstitial tissue and lymphatics. Infection of the alveoli is likewise primarily an interstitial invasion of the alveolar walls by streptococci.

6. In one experiment it was found that preliminary injury to the respiratory tract by gassing with chlorine and that lowering of resistance by a preceding intraperitoneal injection of *Bacillus influenzæ* without local injury to the respiratory tract greatly facilitated invasion of the lungs by *Streptococcus hæmolyticus.*

7. A normal monkey inoculated in the nose and throat with *Streptococcus hæmolyticus* failed to develop pneumonia and showed no evidence of infection of the upper respiratory tract.

CONCLUSIONS.

1. *Streptococcus hæmolyticus* can produce a primary pneumonia in monkeys when injected intratracheally in sufficiently large amounts.

2. It readily produces an extensive secondary pneumonia in monkeys when injected intratracheally in small amounts.

3. Invasion of the lungs by *Streptococcus hæmolyticus* in streptococcus pneumonia in monkeys is primarily by way of the interstitial framework of the lung and its lymphatics, and the disease does not appear to be primarily an infection of the terminal bronchioles.

4. Although it seems probable that invasion of the lungs by *Streptococcus hæmolyticus* in streptococcus pneumonia in man may be by the same paths, it is unsafe to draw this conclusion without qualification, since streptococcus pneumonia in man commonly occurs only as a secondary infection in the presence of a preceding inflammatory bronchitis.

EXPLANATION OF PLATES.

Plate 35.

Fig. 1. Monkey 120. Interstitial pneumonia. Histological section completely through the left lower lobe showing limitation of the lesions to the interstitial framework of the lung. × 3.5.

Fig. 2. Monkey 120. Interstitial pneumonia. Infiltration of perivascular tissue and alveolar walls. × 100.

Plate 36.

Fig. 3. Monkey 119. Interstitial and lobular pneumonia. Posterior aspect of the lungs.

Fig. 4. Monkey 119. Interstitial and lobular pneumonia. Section from the right lower lobe showing exudate in alveolar duct and terminal bronchiole with epithelial desquamation, perivascular infiltration, and exudation into alveoli. × 100.

Plate 37.

Fig. 5. Monkey 119. Interstitial and lobular pneumonia. Proliferation and desquamation of alveolar epithelium. × 400.

Plate 38.

Fig. 6. Monkey 126. Confluent lobular pneumonia. Section from the periphery of the right lower lobe showing edema and infiltration of the walls of atrium and adjacent alveoli with no exudate in the atrium or alveoli. × 100.

Fig. 7. Monkey 126. Confluent lobular pneumonia. Streptococci in an alveolar wall in an unconsolidated portion of the lobe. × 1,000.

Plate 39.

Fig. 8. Monkey 126. Confluent lobular pneumonia. Early lesion of the right upper lobe showing streptococci in the wall of a terminal bronchiole. × 1,000.

Fig. 9. Monkey 125. Confluent lobular pneumonia. Histological section through the right lower lobe. × 3.5.

Plate 40.

Fig. 10. Monkey 125. Confluent lobular pneumonia. Periarterial edema and alveolar consolidation. × 100.

Fig. 11. Monkey 125. Confluent lobular pneumonia. Dense masses of streptococci in perivascular tissue. × 100.

Plate 41.

Fig. 12. Monkey 125. Confluent lobular pneumonia. Early lesion in the right upper lobe showing a point of injury to the epithelium of a large bronchus and leucocytic infiltration and necrosis of the bronchial wall at this point. × 100.

Fig. 13. Monkey 125. Confluent lobular pneumonia. Streptococci in the lesion of the wall of the large bronchus shown in Fig. 12. × 1,000.

Plate 42.

Fig. 14. Monkey 128. Confluent lobular pneumonia. Section from the right lower lobe showing necrosis and dense masses of streptococci about two small vessels. × 100.

Fig. 15. Monkey 48. Confluent lobular pneumonia. Section showing capillary engorgement, perivascular edema, and serous exudate in alveoli with complete absence of polymorphonuclear leucocytes due to leucopenia following a preliminary intraperitoneal injection of *B. influenzæ*. × 100.

Plate 43.

Fig. 16. Monkey 122. Interstitial pneumonia. Section through the right lower lobe showing two pulmonary abscesses. × 3.5.

Fig. 17. Monkey 122. Interstitial pneumonia. Organization and mononuclear cell infiltration of bronchial wall. × 100.

Plate 44.

Fig. 18. Monkey 122. Interstitial pneumonia. Infiltration and organization of interlobular septum and perivascular tissue. × 100.

Fig. 19. Monkey 122. Interstitial pneumonia. Organization of exudate. × 100.

Fig. 1.

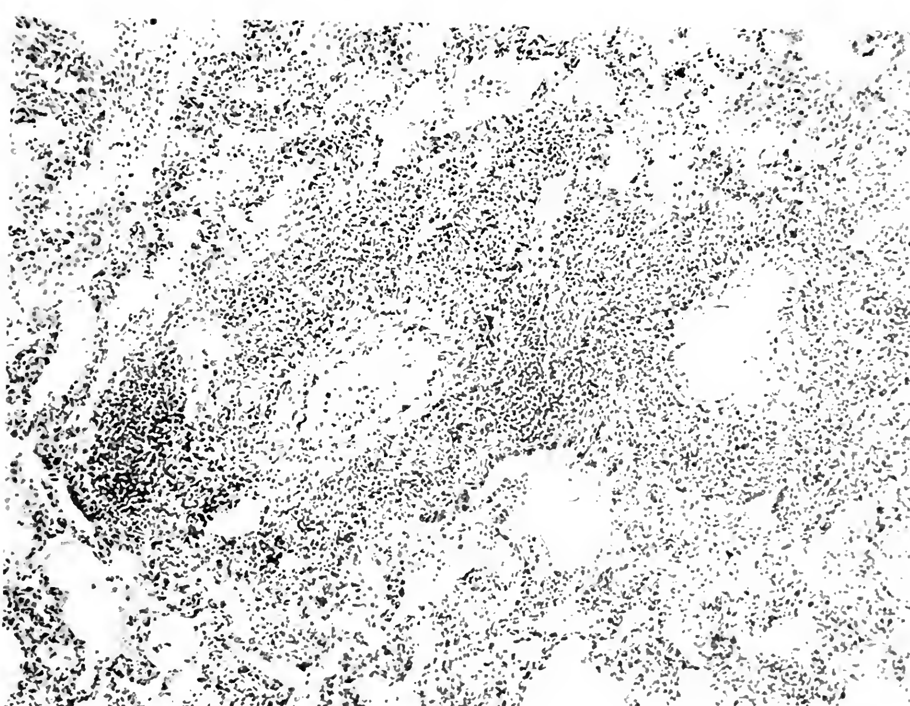


Fig. 2.

Blake and Cecil: Experimental pneumonia. VIII.

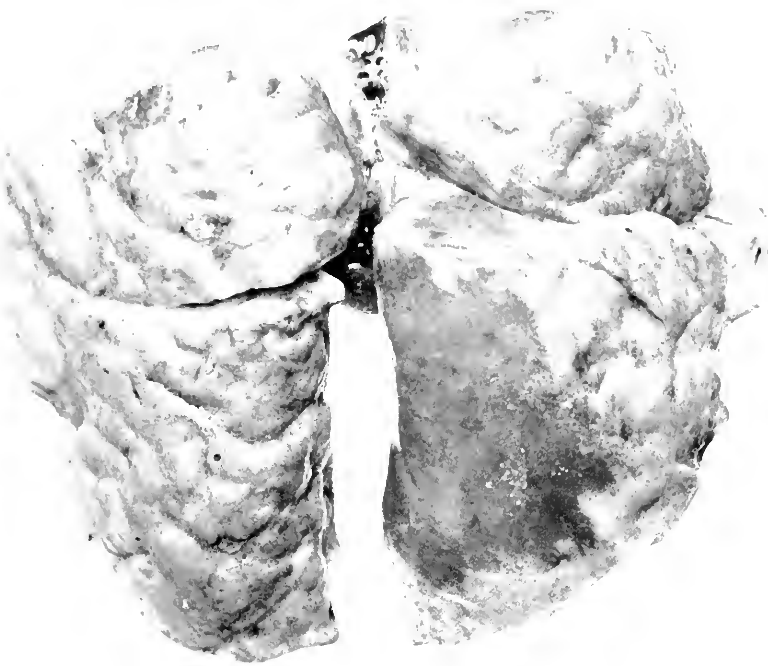


FIG. 3.

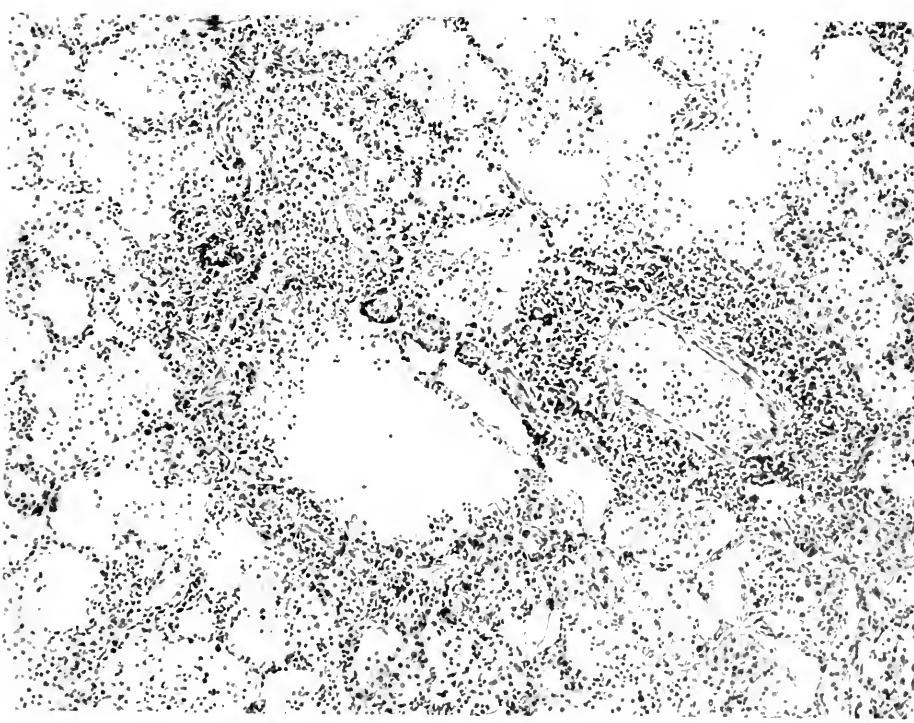


FIG. 4.

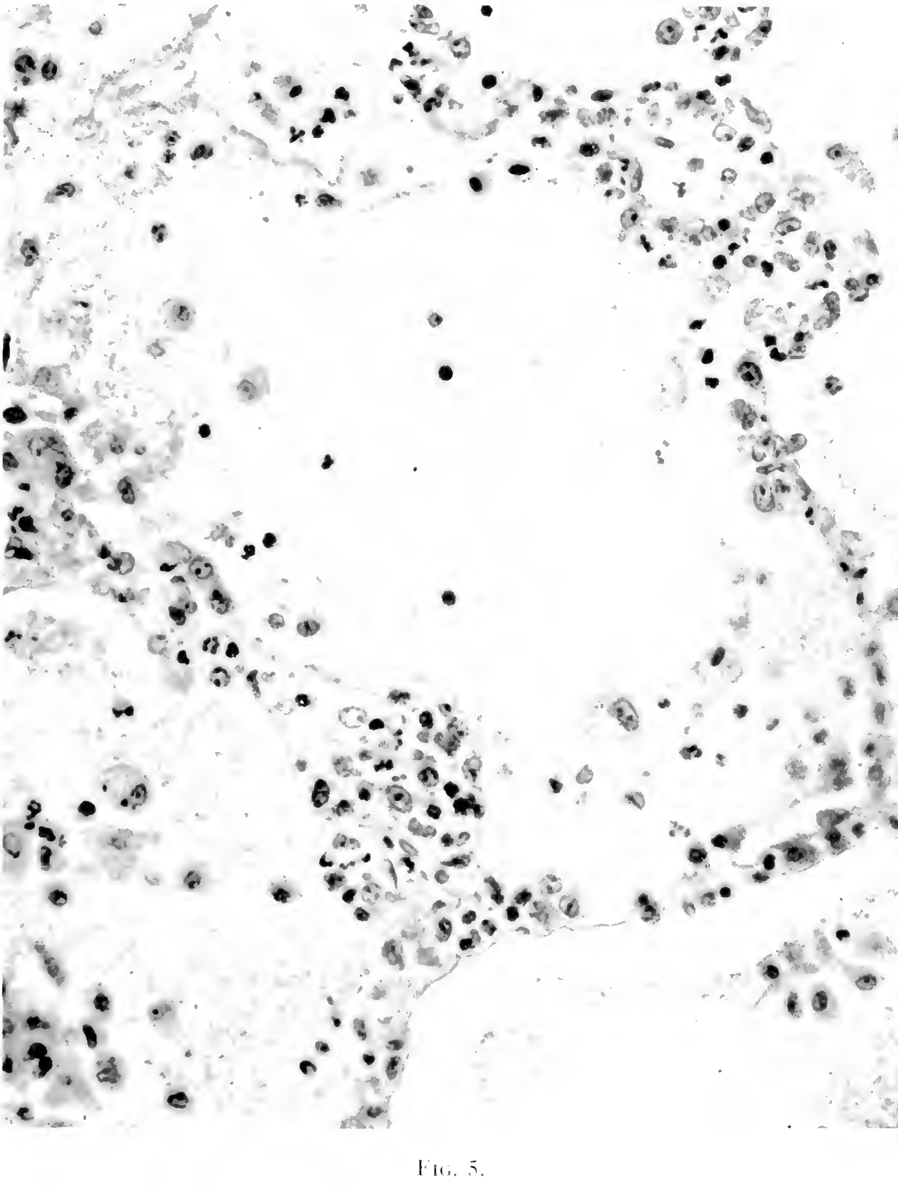


FIG. 5.

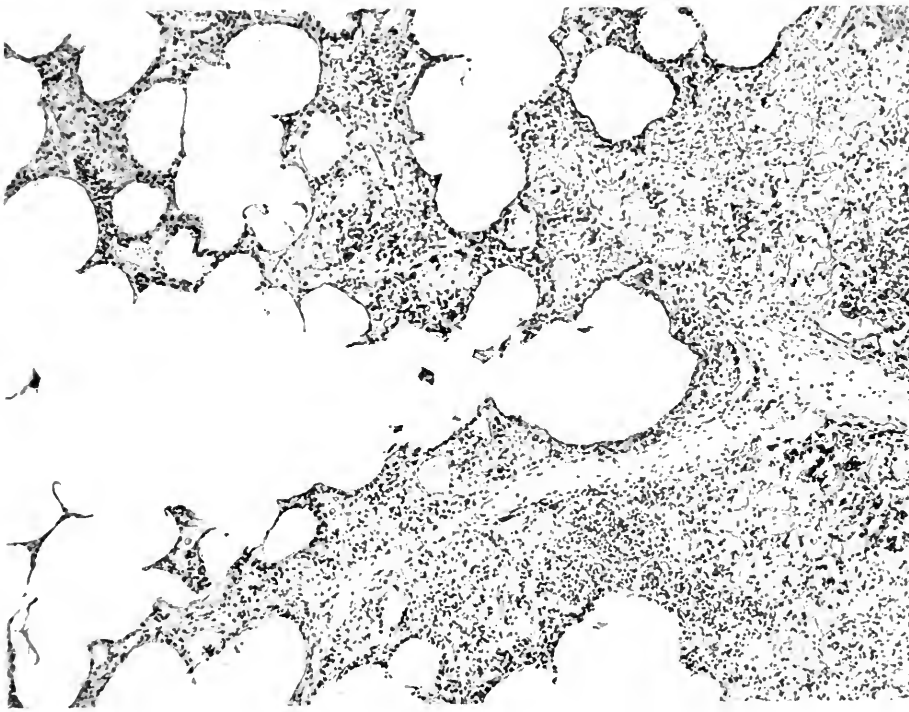


FIG. 6.

FIG. 7.

(Blake and Cecil: Experimental pneumonia. VIII.)

FIG. 8.

FIG. 9.

(Blake and Cecil: Experimental pneumonia. VIII.)

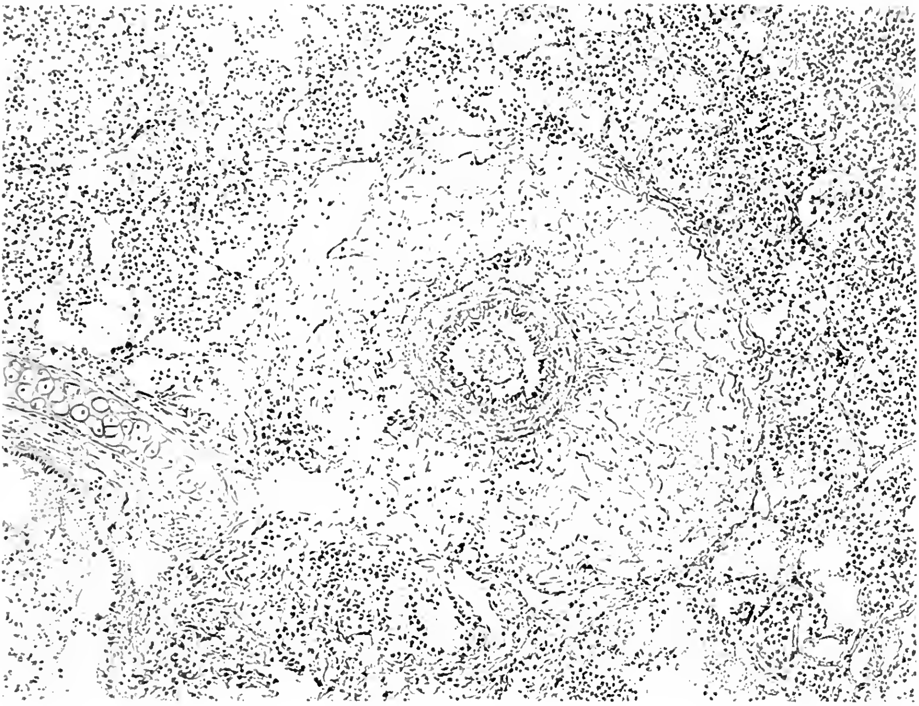


Fig. 10.

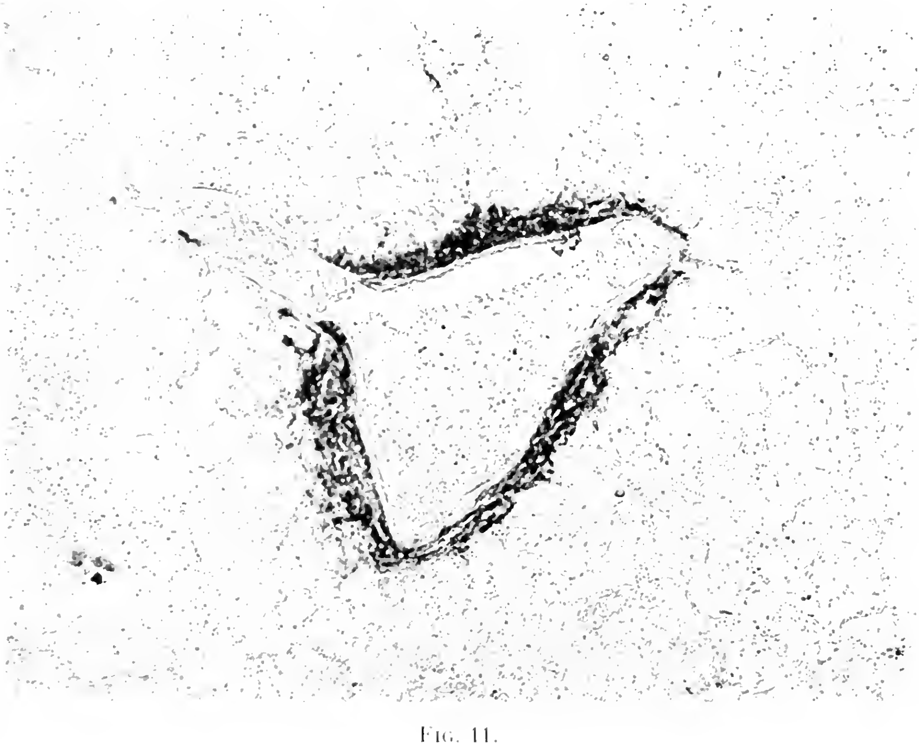


Fig. 11.

(Blake and Cecil: Experimental pneumonia. VIII.)

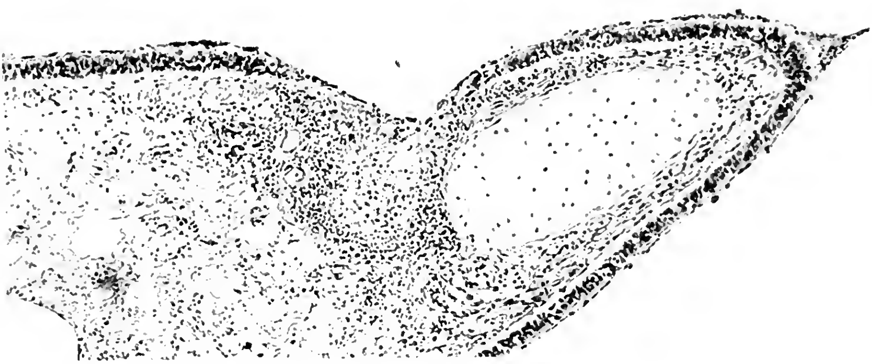


FIG. 12.

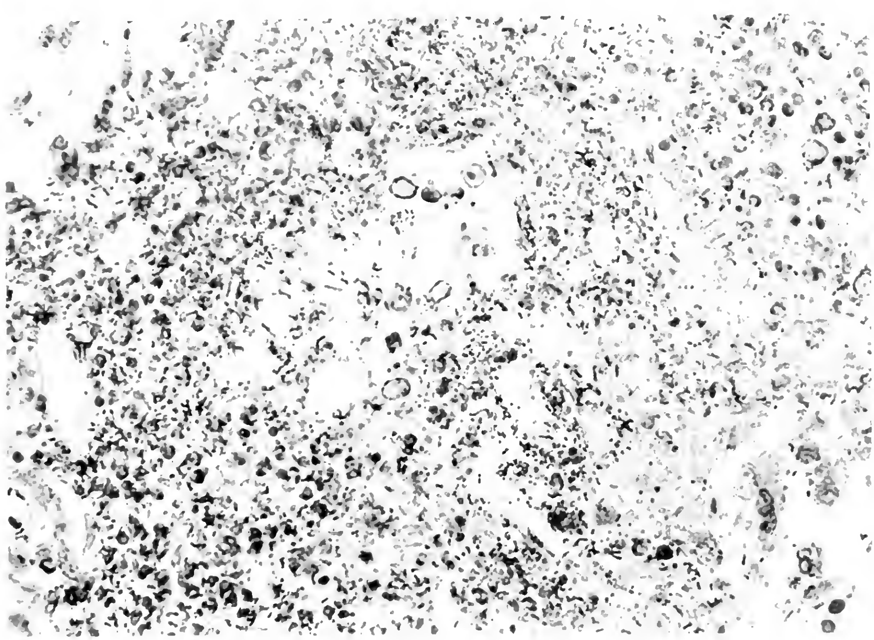


FIG. 13.

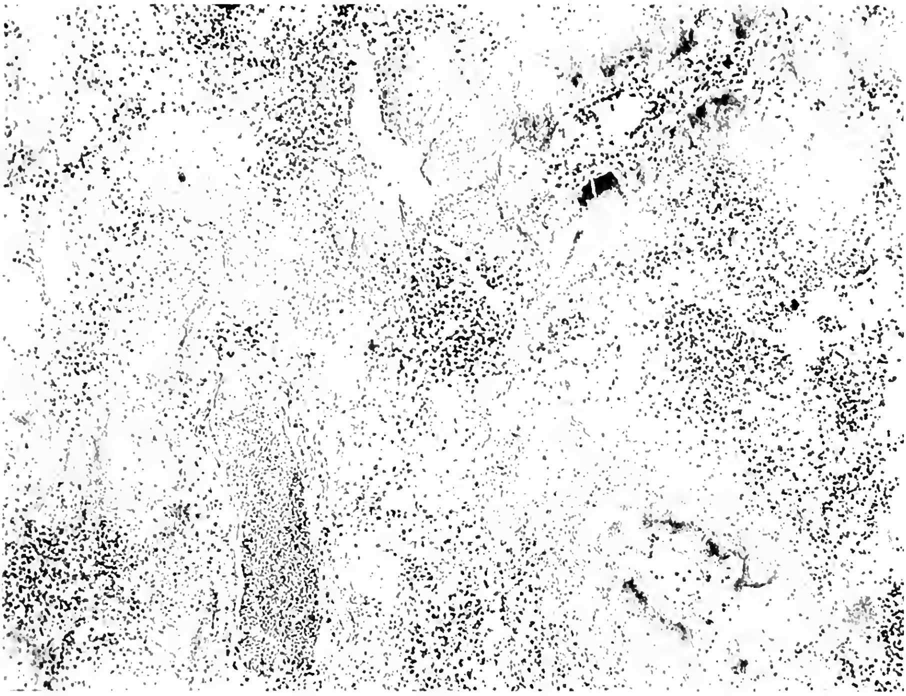


FIG. 14.

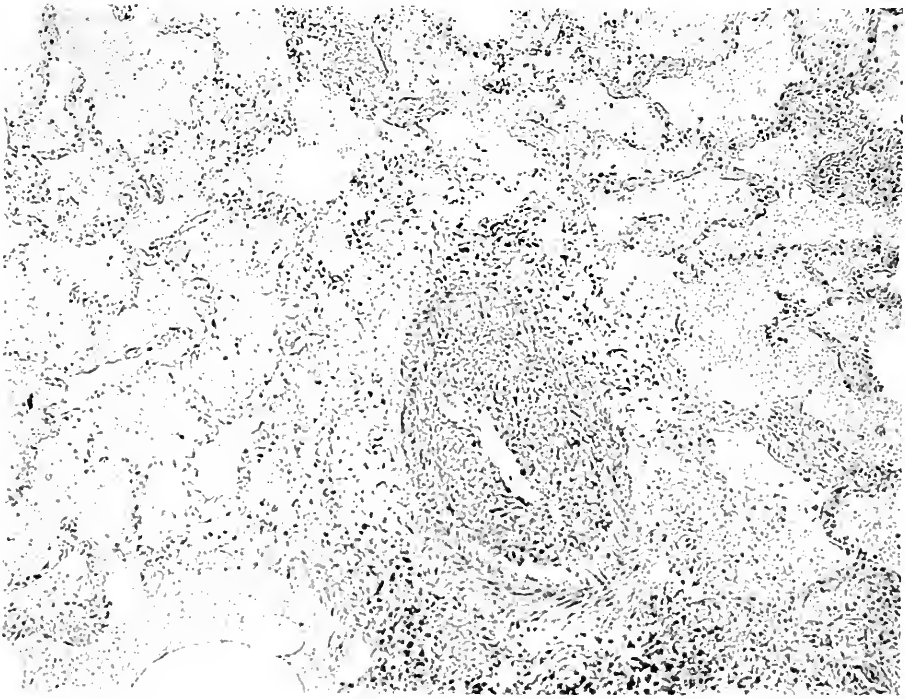


FIG. 15.

(Blake and Cecil: Experimental pneumonia. VIII.)

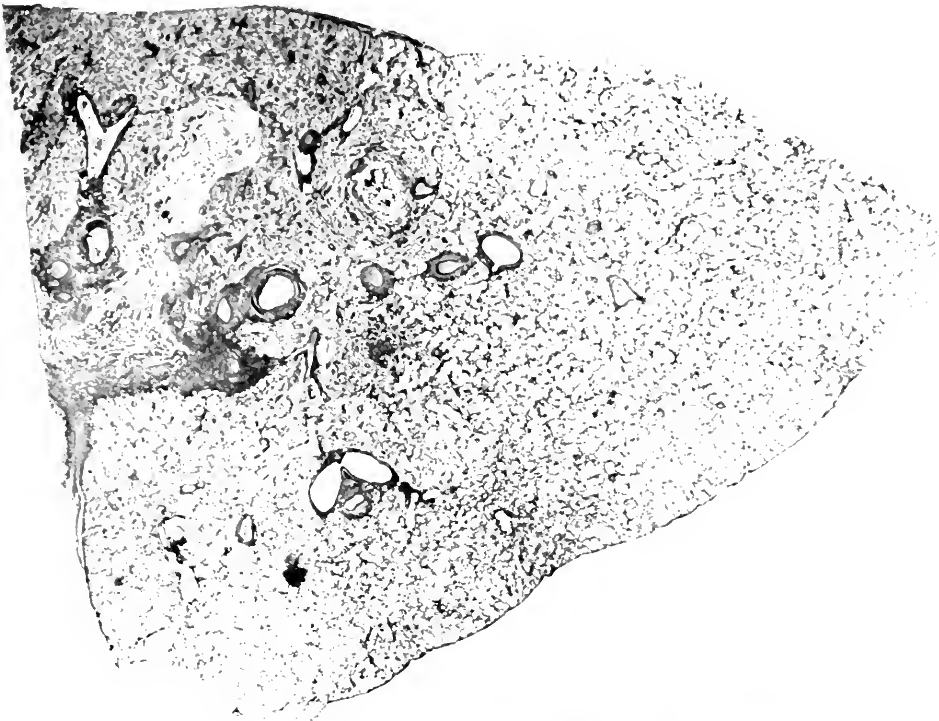


Fig. 16.

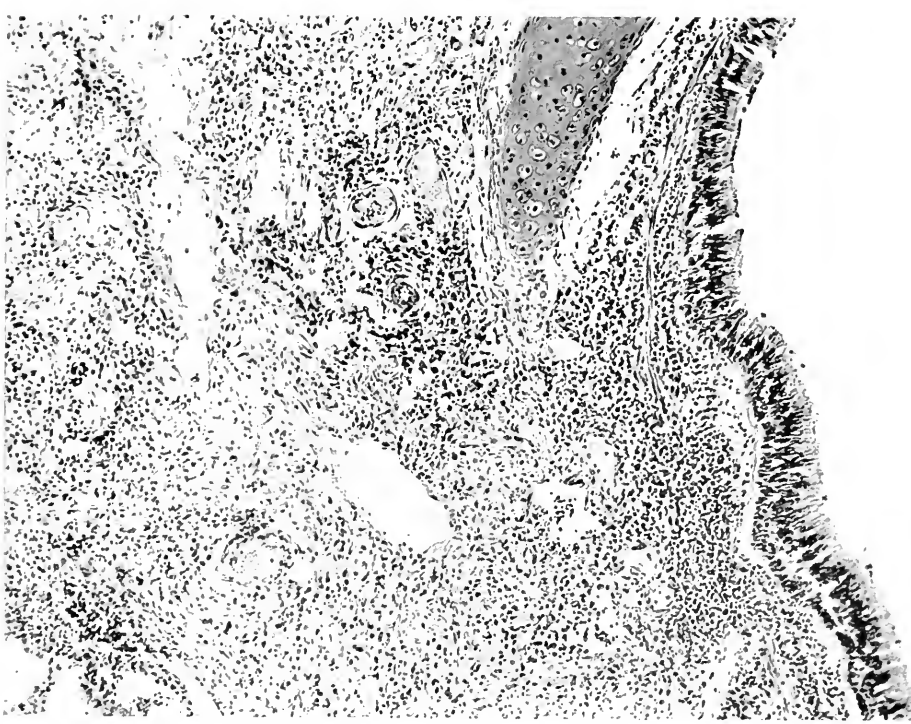


Fig. 17.

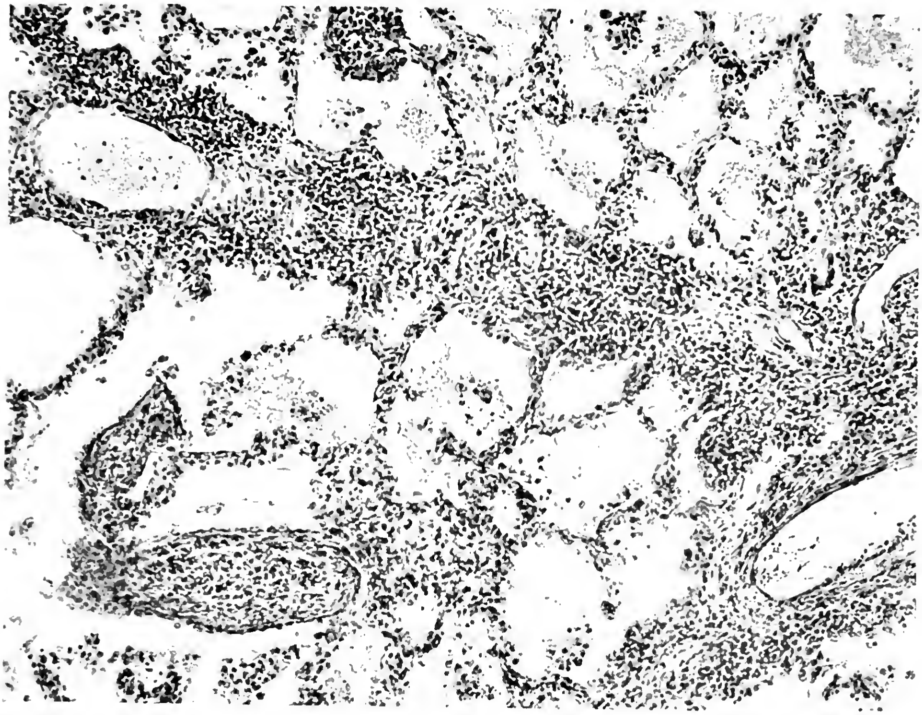


FIG. 18.

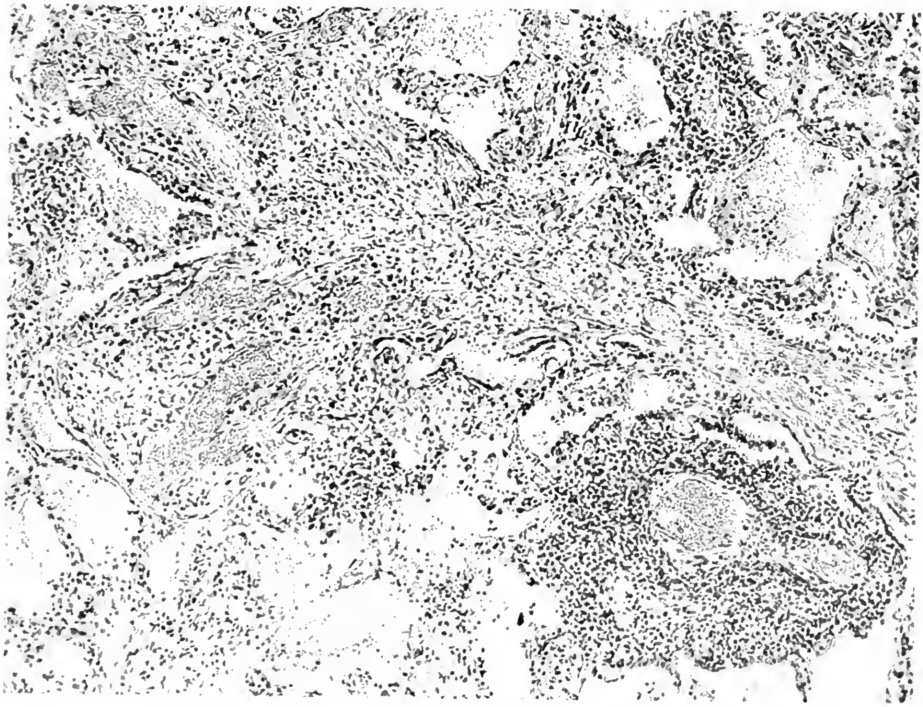


FIG. 19.

(Blake and Cecil: Experimental pneumonia. VIII.)

LOCAL AUTOINOCULATION OF THE SENSITIZED ORGANISM WITH FOREIGN PROTEIN AS A CAUSE OF ABNORMAL REACTIONS.*

By JOHN AUER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 45 and 46.

(Received for publication, June 9, 1920.)

INTRODUCTION.

While testing the sensitiveness of a number of dogs which had been treated with horse serum some years previously, and employing heavy doses of horse serum for the reinjections, it was observed that a peculiar edema developed at the site of the operation wound in the inguinal region. This edema was noted about 2 days after the test and formed a fairly extensive, thick, brawny mass of tissue; there was no discharge from the wound. In order to explain this peculiar type of edema, I assumed that the local reaction was anaphylactic and was produced in the following fashion. In these dogs a foreign protein (horse serum) was circulating, due to the reinjection, and therefore a certain amount of this protein ought to pass into the tissues adjoining the wound during the development of the ordinary wound edema which always follows an operation. Moreover, the amount of foreign protein acting locally would be increased by the oozing of blood, serum, plasma, and lymph into the wound from the severed blood and lymphatic capillary channels, all of which contain the antigen. As the dogs were sensitized to this foreign protein, the skin and adjoining tissues would also be sensitized and could respond by an anaphylactic reaction to this local autoinoculation of the horse serum.

Since this working hypothesis of local autoinoculation could readily be utilized to explain functional changes in any tissue capable of react-

* A preliminary note appeared in the *Proc. Soc. Exp. Biol. and Med.*, 1919–20, xvii. 93.

ing anaphylactically, and since its validity could easily be tested experimentally, and since in addition the conception was new, as far as I was aware, a number of series of experiments were carried out to determine its viability.

EXPERIMENTAL.

Method.

The first tests were made in dogs which were sensitized by the subcutaneous injection of horse serum and then reinjected intravenously after an interval of 4 weeks. At the time of reinjection a deep incision through the skin was made under ether anesthesia with antiseptic precautions, then sutured, bandaged, and the process of healing observed. Although the results were encouraging, it early became evident that practical considerations made the dog an unsuitable test object. For this reason the rabbit was finally selected, and after preliminary tests the skin was again chosen as indicator organ of the reaction. The specific site was the ear, because this could be examined with the maximum of ease and the minimum of discomfort for the animal.

The rabbits were sensitized by four injections of 4 cc. of horse serum at 4 to 5 day intervals. Two injections were given into the erector spinæ muscles and two into the peritoneal cavity. After an incubation period of 15 to 21 days they were reinjected with 10 cc. of horse serum, usually intraperitoneally. In one series half the reinjection dose was given into the erector spinæ muscles and half into the peritoneal cavity. The intravenous route for reinjection was not employed because a test showed a large number of fatalities in the sensitized group. Thus in Series 2 four out of six sensitized rabbits died in 2 to 15 minutes after the intravenous injection of 5 cc. of horse serum, which made this series valueless.

The initial local damage to provoke a local edema and inflammation was produced by painting the external surface of the ear with 1 cc. of xylol and rubbing it gently for 15, 20, or 30 seconds, the time interval depending upon the series. This skin irritant causes a marked temporary flushing of the ear vessels which is followed by a mild inflammation and edema of varying degree. It will be noticed that this skin irritant was used to bring about the same condition as the skin incision in dogs, the production of a certain degree of inflammation and exudate into the tissues. In Series 1 a chemically pure *o*-xylol (Kahlbaum)[1] was used; in the others a commercial xylol (Merck) was employed.

Commercial xylol was chosen for the later series because the chemically pure isomers were available only in small amounts and also because the commercial

[1] I am indebted to Dr. W. A. Jacobs for supplying me with some of Kahlbaum's *o*-, *m*-, and *p*-xylol. It may be mentioned that *o*-xylol is more effective in producing edema than *m*- or *p*-xylol.

product seemed to be more effective in producing a local edema. Since the commercial product varies in its percentage composition of *o*-, *m*-, and *p*-xylol, and since it probably also contains various impurities, commercial xylol (Merck) from the same container was used throughout the subsequent series.

The same dose of 1 cc. of xylol was given to all rabbits and was always measured by the same pipette.

The xylol was usually applied to the skin 30 to 45 minutes after the reinjection of horse serum; in one series the interval was 3 hours. These intervals were allowed to pass in order to permit the absorption of a certain amount of horse serum, so that any edema developing after the xylol application would contain some of it.

The rubbing of the ear was always gentle and was done with a finger covered by a rubber cot. As the ear had to be held during the rubbing, some of the xylol always moistened the finger on the inner surface of the ear. To a certain extent, therefore, xylol was applied to the outer and inner ear surfaces, though by far the most was received by the external surface. The time during which the ear was rubbed was always controlled by a clock (15, 20, or 30 seconds, depending upon the series).

For each sensitized and reinjected rabbit at least two different control experiments were carried out at the same time. In one series of controls the normal rabbits received 10 cc. of horse serum intraperitoneally 30 to 45 minutes before treating the ear with xylol (serum controls). In the second series of controls no horse serum was administered, and the only interference was painting the ear with xylol (ordinary controls). In a third group of controls the rabbits were sensitized with horse serum, and after the incubation period they were not reinjected, but xylol was applied to the ear (sensitized controls). In general each series of experiments consisted of 18 rabbits: 6 sensitized and reinjected; 6 horse serum controls; and 6 normal controls.

The horse serum[2] employed was always at least 4 months old. In the earlier series the serum was sterile and without any preservative; in later series the serum contained chloroform. When chloroform was present this was driven off by floating a sterile evaporating dish containing the serum in a dish of warm water. As far as possible the same lot of serum was used for sensitizing and reinjection.

The injections were always made with a sterile syringe, and the site of puncture was prepared by cutting the hair and treating the area with dilute tincture of iodine.

All the rabbits were examined first at 2 to 4 hour intervals; then daily; later at 2 to 3 day intervals. The entire period of examination lasted about 2 weeks when no reaction was obtained; if a reaction took place the examinations were usually continued for 3 or 4 weeks.

Most of the rabbits employed were males; when females had to be used the same number of females was added to each group in a series. All were kept in

---

[2] The horse serum was obtained from the New York City Board of Health through the kindness of Dr. W. H. Park, Dr. E. J. Banzhaf, and Dr. I. Greenwald.

individual cages. The weight fluctuated between 1,400 and 2,000 gm. The diet consisted of oats, hay, cabbage, and turnips; occasionally carrots were available. The ordinary gray variety formed the majority; no white rabbits were used because they could not be obtained in sufficient numbers. As far as possible young adult animals were employed; very young rabbits were never used.

Results.

Before describing the experimental results more fully, it may be stated at once that the work strongly supports the working hypothesis upon which the investigation is based. Thus in three series of experiments, totalling 53 animals, the xylol-treated ears of 36 controls showed no dermatitis with blisters and crust formation; nor were hemorrhages and gangrene observed except in one and two instances respectively. In the latter instances the loss of substance did not exceed 0.5 mm. of the ear tip. The ears of these controls were practically normal 3 to 4 days after the xylol application.

The picture was, however, quite different in the sensitized and reinjected groups. Out of seventeen rabbits, ten developed an exfoliative dermatitis a few days after the xylol treatment. The blisters and crusts covered one-third to one-half of the ear surfaces, involved the deeper tissues, and always led to dry gangrene in these particular animals. The gangrene caused a loss of ear substance at the tip, varying from 1 to 3 cm. Healing was slow, but usually complete in 3 to 4 weeks. The ear stumps were at first bald, with a glistening, thin skin; later a new growth of white hair appeared (Figs. 1 to 3).

The ear changes may now be considered more in detail. When 1 cc. of *o*-xylol or commercial xylol is applied to the hairy surface of the rabbit's ear and then gently rubbed for 15 or 30 seconds, a marked dilatation of the blood vessels is usually produced fairly promptly. This flushing of the ear lasts longer than 10 minutes, but has usually disappeared after 45 minutes. At this time the ear generally presents a faint pink flush and the vessels are narrow. After 3 to 6 hours a fair to moderate edema appears in a majority, but not in all of the treated ears in the two control groups. Within 48 hours the edema in these two groups has practically disappeared in most animals, and in 3 days the treated ears are largely normal, if we exclude a slight desquamation, a slow loss of hair which is promptly replaced by normally pig-

mented hair, and a tendency of the ear arteries to flush more readily and more strongly than in the untreated ear (Table I).

TABLE I.

Onset and Course of Edema in Series 1, 3, and 4.

Time.	Sensitized and reinjected rabbits (17).	Horse serum controls (18 rabbits).	Ordinary controls (18 rabbits).
hrs.			
3–6	No or slight edema, 14. Slight to fair edema, 3.	No or slight edema, 5. Fair to moderate edema, 13.	No or slight edema, 7. Fair to moderate edema, 11.
22–27	No or slight edema, 10. Fair to moderate edema, 1. Moderate to marked edema, 6.	No or slight edema, 11. Fair to moderate edema, 6 (1 died).	Slight edema, 16. Moderate " 1. Marked " 1.
48	No or slight edema, 11. Fair to moderate edema, 3. Marked edema, 3.	No or slight edema, 14. Fair edema, 2. Moderate edema, 1.	No or slight edema, 16. Fair edema, 1. Moderate edema, 1.
72	No or slight edema, 11. Moderate edema, 2. Marked edema, 3 (1 died).	No or slight edema, 16. Slight to fair edema, 1.	No or slight edema, 16. Fair edema, 2.
120	No or slight edema, 13. Marked edema, 3.	No or slight edema, 14. Marked edema,* 3.	No or slight edema, 18.

* This recurrent edema practically disappeared the next day and did not reoccur. See text for details.

When the xylol edema has disappeared there is usually no recurrence of it. Only three exceptions were noted in the large number of controls, and they occurred in the horse serum control group of Series 1. These three rabbits had received horse serum for the first time about 45 minutes before the ear had been treated with *o*-xylol (Kahlbaum). A fair edema developed and disappeared after 48 hours. On the 3rd day none of them showed any edema (Table I). On the 5th day (no observation made on the 4th) all three showed a strong edema which was more marked than that seen previously. The next day (6th), however, the edema had practically disappeared in all, and no further edema was noted during the next 9 days, when observations were discontinued. No exudate ac-

companied this recurrent edema, nor did any gangrene take place. One of these rabbits also showed numerous small discrete hemorrhages scattered on the central portion of the upper dorsal half of the ear.

No blisters or crusts were observed in the great majority of both control groups. In the large number of controls only two cases were seen. These two animals belonged to the ordinary controls of Group 3 and had therefore never been subjected to any serum action. The exudate which they showed was very slight, occupied the extreme tip of the ear, and the subsequent dry gangrene of the

TABLE II.

Dry Gangrene of the Ear Tip.

Series No.	Sensitized and reinjected rabbits.	Horse serum controls.	Ordinary controls.
Series 1 (*o*-xylol).	Gangrene, 4.* No gangrene, 2.	Gangrene, 0. No gangrene, 6.	Gangrene, 0. No gangrene, 6.
Series 3† (commercial xylol).	Gangrene, 3. No gangrene, 2.	Gangrene, 0. No gangrene, 6.	Gangrene, 2.‡ No gangrene, 4.
Series 4 (commercial xylol).	Gangrene, 3. No gangrene, 3.	Gangrene, 0. No gangrene, 5 (1 died).	Gangrene, 0. No gangrene, 6.

* The loss of substance in all three groups varied from 10 to 30 mm.

† Series 2 was lost through death of most of the sensitized group due to intravenous reinjection.

‡ The loss of substance was less than 1 mm. of the ear tip.

tip was less than 1 mm. (Table II). The bald tip of these ears later grew a small tuft of unpigmented, white hair.

In the sensitized and reinjected series of rabbits the application of xylol to the ear produces in general the same immediate effects as those noted for the control group, except that the flushing of the ear vessels at times is not quite so prompt in the animals tested 30 to 45 minutes after the horse serum injection; in Series 5, however, in which 3 hours passed before the xylol was applied to the ear, all ten rabbits showed a prompt and strong initial vasodilatation. A similar prompt initial flushing after xylol was also obtained in the group of four

animals which were sensitized but not reinjected. The development of the pink diffuse blush which preceded the appearance of a definite edema was delayed in the reinjected series as well as in the horse serum controls of Series 3 and 4; in Series 1, however, all the rabbits, both the reinjected as well as the two control groups, showed a diffuse pinkness $2\frac{1}{2}$ hours after the xylol application.

A difference between the reinjected groups and the control groups of all the series was shown in the development, degree, and persistence of the edema called forth by the xylol. Table I summarizes these variations. From this table it will be seen that the edema in some rabbits of the sensitized reinjected groups developed more slowly, persisted longer, and reached a greater degree than in either of the control groups. The table also indicates that a somewhat larger percentage of the sensitized reinjected group apparently never passed beyond a slight degree of edema than in either of the control groups.

The most striking difference, however, between the sensitized reinjected groups and the control groups was exhibited in the development of blisters, crusts, and dry gangrene in the xylol-treated ears (Figs. 1, 2, and 3). These lesions occurred practically only in the sensitized reinjected groups, and in them ten out of seventeen rabbits showed extensive lesions. In the control groups, on the other hand, only two rabbits out of thirty-five (serum controls + ordinary controls) showed a slight crust formation at the ear tip which was followed by a minimal loss of tissue (Table II).

The blisters usually were observed in 24 to 48 hours; in one instance only was a bleb seen 6 hours after the xylol application. In typical cases the blisters appear first on the internal surface of the ear along the medial or lateral border. On the external surface usually a dry brownish black crust is first seen. The blisters appear in crops, varying in size from 2 to 4 mm. in diameter; in one instance a single large, thick walled blister 20 by 12 mm. occurred at the tip of an ear. The walls of the blisters are usually fairly thin and the blisters are at first filled with a yellowish fluid which later turns brown and forms a brownish black crust on drying. Some of the blisters also contained blood. In some instances no blisters were seen but exudate and soft crusts were the first alteration noticed. Thus extensive soft crusts were

occasionally seen for the first time 5 days after the xylol application. These crusts may be extensive and approximately one-half of the ear may be covered with them. Along the borders of the ear the crusts always extend farther towards the root than along the middle of the ear. Removal of some crusts showed that the entire skin was involved, for a raw bleeding surface was left exposed (Fig. 2).

Within 3 to 5 days the upper portion of the ear began to fold and curl slightly, and in 7 to 10 days the tip of the ear was black, dry, and hard. Separation of the gangrenous portion took place slowly and usually occurred 11 to 14 days after the xylol application, at which time the process was hastened mechanically. The loss of substance varied in the three series under discussion from 10 to 30 mm. of the tip of the ear (Figs. 1, 2, and 3). The loss of substance along the lateral border was well shown by the fact that the marginal ear vein formed part of the gangrenous area or now appeared within a millimeter or less of the edge of the ear for a portion of its course.

Removal of the dry gangrenous tip and crusts left the upper ear practically bald; the skin was thin and glistening with a few raw and slightly bleeding surfaces; the borders were thickened, nodular, with some tongue-like projections. The entire upper ear was moderately edematous, of a diffuse bluish pink color, and the vessels were moderately blurred on transillumination, probably due to the edema of the surrounding tissues. Healing was usually complete in 3 to 4 weeks.

After 2 weeks the surface of the ear stump gradually became covered with hair, but the new growth of hair was entirely devoid of pigment. This white hair has now persisted without any change for more than 5 months in two typical cases which were allowed to survive.

No effort was made to follow the histological changes of the ear lesions at various stages of their development because this would have entailed the loss of too many animals and at present it was considered more important to observe the gross changes.

One more difference remains to be noted, the occurrence of numerous small discrete hemorrhages on the dorsal surface of the xylol-treated ears of sensitized reinjected rabbits. The fact that the presence of numerous small hemorrhages is being considered should be emphasized at once, for the appearance of an occasional small hemorrhagic spot or two is not infrequent in the ears of apparently normal rabbits. The petechial hemorrhages under discussion were most frequent

in the sensitized reinjected rabbits of Series 1 in which four out of six rabbits exhibited them. Among the twelve controls of this series only one animal, a serum control, developed similar hemorrhages. In Series 3 only one animal showed these hemorrhages, and it was a member of the sensitized reinjected group; the twelve controls showed no hemorrhages. In Series 4 only two rabbits developed petechiæ of the ear, and they again belonged to the sensitized reinjected group; in this series also the twelve controls showed no hemorrhages. To sum up, therefore, petechial hemorrhages occurred in seven out of seventeen sensitized and reinjected rabbits, but only once in thirty-six controls; they were especially frequent in Series 1 (four out of six) and infrequent in Series 3 and 4 (one or two out of six). This difference in frequency of occurrence in the three series is possibly related to the fact that in Series 1 chemically pure *o*-xylol (Kahlbaum) was employed, while in Series 3 and 4 commercial xylol (Merck) was used.

Supplementary Experiments.

Three other series of experiments were carried out for reasons which will be considered later. In the first of these, Series 5, the effect of allowing 3 hours to elapse between the injection of horse serum and the application of xylol was investigated. This series was composed of ten rabbits all of which had been sensitized, but not to the same degree. The number of sensitizing doses given were as follows: 6 injections, 2 rabbits; 2 injections, 3 rabbits; and 1 injection, 5 rabbits. All these animals had been used previously; two had been members of a sensitized reinjected group and the other eight had served as serum controls. 21 days had passed since the last serum injection and the last xylol application. On the day of the test each rabbit received 10 cc. of horse serum intraperitoneally; after 3 hours, none of them having shown any signs of collapse, the left ear was treated with 1 cc. of commercial xylol and gently rubbed for 15 seconds. It should be added that the left ear had not been subjected to the action of xylol in previous tests.

Since these animals form three groups of varying degree of sensitization, it is perhaps of little value to compare them in detail with the series previously described, which again have another degree of sensitiveness. The main point is that one rabbit in each set, that is, three rabbits in all, developed a marked exudate and crust formation on the xylol-treated ear. These crusts were first noted in one rabbit on the 4th day and in the other two rabbits on the 7th day (no obser-

vations unfortunately were made between the 4th and 7th days). The crusts involved both ear surfaces, varied in extent from 2 to 5 cm., and were in all respects apparently similar to those observed in Series 1, 3, and 4. The resultant gangrene, however, was much less than that observed in the earlier experiments; one rabbit lost 6 mm. of the ear tip, and the other two suffered but a slight loss of ear tissue. In the last two cases the bald ear tip was bluish, puckered, and considerably thickened, in one instance to 3 mm. About 3 weeks after the application of the xylol, and 2 weeks after the removal of the crusts, the bald ear tips showed a new growth of white hair.

For the sake of completeness it may be added that after 6 hours the edema produced by the xylol application was only slight in seven rabbits and between slight and fair in three. In the latter three rabbits, the ear later developed blisters, exudate, and crusts as described above. After 48 hours the edema had disappeared in practically all. These rabbits, therefore, as far as delayed onset of the edema and development of a severe dermatitis were concerned, resemble the sensitized reinjected groups of previous series; they differ, however, from the latter in the relatively short duration of the edema which was produced.

The same animals, with the exception of one which was killed, were again used 22 days later in order to test the effect of xylol on the ears of sensitized but not reinjected rabbits. The rabbits therefore were only subjected to the action of 1 cc. of commercial xylol applied to the same ear which had served in the previous test; the ear was then gently rubbed for 15 seconds. An edema was noticed after 2 hours which increased moderately after 6 hours and practically disappeared after 24 hours. No blisters, crusts, hemorrhages, or gangrene of any degree were observed during the next 13 days, at the end of which time the animals were discarded. It will be observed that this group of sensitized but not reinjected rabbits behaved like ordinary normal control rabbits.

In order to verify still further the action of xylol upon sensitized but not reinjected rabbits, five normal animals were each sensitized by four subcutaneous injections of 1 cc. of horse serum, at 3 to 6 day intervals. When 21 days had elapsed after the last serum injection the left ears of the four survivors[3] were treated with 1 cc. of com-

[3] One rabbit died during the process of sensitization.

mercial xylol, and then lightly rubbed for 15 seconds. All the ears flushed promptly and strongly on application of the xylol; after $1\frac{3}{4}$ hours all showed a fair to moderate edema which increased up to 6 hours after the xylol treatment. 22 hours afterwards the edema in all had decreased, and in 48 hours it had practically disappeared. These rabbits were examined daily for 14 days and at no time were any hemorrhages, blisters, or crusts seen. In this group again the sensitized but not reinjected rabbits had behaved like the ordinary control groups.

DISCUSSION.

It has been clearly demonstrated above that a mild skin irritant, such as xylol, produces strikingly different effects when applied to the ears of control rabbits or to the ears of rabbits previously sensitized and reinjected with horse serum. In normal as well as in serum control animals or rabbits which have been sensitized but not reinjected, the xylol produces only a moderate inflammation with edema, and no blistering or obvious gangrene results. In sensitized and reinjected rabbits, on the other hand, xylol exhibits in a majority of the rabbits a marked blistering effect, followed by heavy crust formation and tissue destruction; the tissue destruction often leads to a dry gangrene involving several centimeters of the ear tip. It is thus seen that xylol acts like a rubefacient in the three types of controls, while in the sensitized and reinjected rabbits the same agent, applied in the same dose and in the same way, behaves like a vesicant and escharotic.

In an interpretation of these findings the most obvious, indeed the only explanation is that an anaphylactic reaction plays a dominant part, since the lesions were observed in sensitized and reinjected rabbits only. The ear lesions, therefore, may be the direct expression of a local anaphylactic reaction, as the working hypothesis postulates, or they may be the secondary result of an anaphylactic reaction or reactions taking place elsewhere than in the ears.

In an analysis of the view that the lesions are the secondary result of anaphylactic reactions the general reactions which come into consideration are (1) an anaphylactic fall of general blood pressure; (2) local changes in the ear circulation due to general anaphylactic reaction; (3) anaphylactic abnormalities of cardiac action; and (4) anaphylactic changes in the blood.

Fall of Blood Pressure.[4]—Though no blood pressure determinations were made for obvious reasons, it can be definitely stated that a profound drop did not occur. This is demonstrated by the fact that none of the sensitized rabbits showed a definite prostration or collapse after the reinjection, a condition which always accompanies a severe lowering of the blood pressure in anaphylactic rabbits. If a fall of blood pressure occurred, it therefore must have been at most a moderate drop. Concerning the duration of this hypothetical blood pressure fall, indirect evidence must be sought from experiments in which the reinjected dose was given intravenously, for there is no record of blood pressure studies in the anaphylactic rabbit when the reinjection is carried out by way of the peritoneal cavity, which is the route employed in the experiments reported in this paper. Now it may be justly assumed that the blood pressure fall of sensitized rabbits is surely not severer or of longer duration in rabbits reinjected peritoneally than in similarly sensitized rabbits in which the intoxicating dose is administered intravenously. I have numerous instances of the latter type from earlier experiments,

TABLE III.

Blood Pressure Fall of Sensitized Rabbits Reinjected Intravenously.

	Normal blood pressure.	Lowest level after injection.	Recovery level.	Time required for recovery level.	Dose of serum.
	mm.	*mm.*	*mm.*	*min.*	*cc.*
1	110	56	103	20	10
2	104	63	103	30	—
3	118	80	110	12	10
4	116	51	108	25	10
5	130	85	108	17	20
6	120	79	110	6	10

and Table III illustrates the degree of blood pressure fall and the time required to regain a practically normal level. These rabbits were sensitized by repeated (spaced) injections of horse serum at intervals, and were reinjected intravenously with 10 cc. of the same serum. The blood pressure was recorded with a Hürthle membrane manometer. Many mates of these animals succumbed acutely to the same reinjection dose.

Table III illustrates how promptly, within 6 to 30 minutes, well sensitized rabbits may regain a practically normal blood pressure level even though the anaphylactic blood pressure drop is severe.

It must be remembered that the xylol was never applied to an ear until at least 30 to 45 minutes had elapsed after the intraperitoneal injection of 10 cc. of horse serum; in one series (No. 5) 3 hours were allowed to pass. It may then be concluded with a fair degree of certainty that all the animals had recovered

[4] Arthus, M., *Arch. internat. physiol.*, 1908–09, vii, 479.

largely if not entirely from the anaphylactic fall of blood pressure before the xylol was applied to the ear. The anaphylactic fall of blood pressure, therefore, may be disregarded as a factor in the production of the ear lesions.

Local Changes in the Ear Circulation.—A factor of perhaps greater importance than a fall in the general blood pressure is the degree of circulation which is maintained in the xylol-treated ear. The circulation in the treated ears of the sensitized reinjected rabbits might be less efficient than in the xylol-treated ears of controls. This factor was controlled as far as possible by inspection at $2\frac{1}{2}$, 3, and 6 hour intervals after the application of the irritant. There was no significant change in the vascularity of a majority of the ears in the sensitized reinjected groups which was not also present in a majority of one or both control groups at the same time. One factor perhaps indicates that the circulation in the xylol-treated ears of the sensitized reinjected group was not so good as in the control ears; *viz.*, the fact that the edema developed more slowly than in the control group. It is also possible, however, that this delay in the onset of edema is the result of blood changes which will be touched upon later. On the whole, however, there is no definite evidence that the circulation is poorer in the sensitized reinjected group of rabbits after the application of xylol than in the control ear.

Abnormalities of Cardiac Action.[5]—These are very frequent in anaphylactic rabbits after intravenous injections but their duration is relatively short. As a rule they last only 7 to 21 minutes after the reinjection. Consequently, even if it is granted that the same cardiac changes appear in sensitized animals after intraperitoneal reinjection, the animals had ample time to recover before the xylol test was made (30 to 45 minutes and 3 hours after the serum injection).

Anaphylactic Changes in the Blood.[6]—What effect variations in the coagulability of the blood, alterations in the blood picture, and other anaphylactic hemic changes have upon the resistance of body cells is not known, and their influence in the production of the xylol ear lesions cannot therefore be estimated. As mentioned before, these factors may perhaps be the cause of the delayed onset of the edema in the sensitized reinjected animals.

An observation which deserves mention is that the xylol-treated ears of the sensitized and reinjected group often remained cooler than the non-treated ear of the same animal for some hours; after this period they became warmer than the non-treated ear. The treated ears of the control groups, on the other hand, usually were warmer than the non-treated ears within 2 hours after the application of the xylol. There was, however, no relation between the incidence of blisters, skin destruction, and gangrene and this initial coolness of the ear; the lesions

---

[5] Auer, J., and Robinson, G. C., *J. Exp. Med.*, 1913, xviii, 450.

[6] Arthus, M., *Arch. internat. physiol.*, 1910, ix, 157. von Pirquet, C., and Schick, B., Die Serumkrankheit, Leipsic and Vienna, 1905. Schlecht, H., and Schwenker, G., *Deutsch. Arch. klin. Med.*, 1912, cviii, 405. Weinberg, M., and Séguin, P., *Compt. rend. Soc. biol.*, 1914, lxxvi, 585.

occurred and failed to occur in ears which had been cooler than the untreated ears for the first few hours; similarly they appeared or failed to appear also on ears which had been warmer than the untreated ear of the same animal. There was apparently, however, a definite relation between the initial coolness of the treated ear in the sensitized reinjected group and the subsequent degree of edema; if the ear was initially cool for some time, the edema which developed later (before the onset of skin inflammation) was generally slight. From the facts stated above it follows that the degree of edema bears no relation to the incidence of ear lesions. Typical lesions of the treated ear in the sensitized reinjected groups occurred after slight, as well as after marked edema.

From this survey it may be concluded that none of the factors discussed offers a well founded, satisfactory explanation of the ear lesions; in spite of this, however, it is possible that some, or perhaps even all may play a part in the circle of events which are necessary to produce the end-result.

We come now to the alternative explanation, that the ear lesions are the expression of a primary, local, anaphylactic reaction. This interpretation is the one upon which the investigation was founded and which was briefly stated in the beginning of this paper. The working hypothesis assumed that a sensitized organism, under certain conditions, could reinject itself locally with the same antigen which caused sensitization, thus producing the state necessary for an anaphylactic reaction in that locality. The conditions were (*a*) the presence of the antigen in the circulating juices of the sensitized organism, and (*b*) the local accumulation of this antigen in a tissue showing some degree of inflammation.

These conditions were met experimentally by employing rabbits sensitized with horse serum, and, after an incubation period, reinjecting them intraperitoneally with a readily tolerated dose of the same serum. After a time the blood and lymph would necessarily contain some of the horse serum due to absorption from the injection depot. The local accumulation of the antigen in an inflamed area (condition *b*) was brought about by the application of a mild skin irritant, xylol, upon the ears of the rabbits. As this irritant causes inflammation and edema of the ear tissues, it is obvious that the edema fluid would contain more or less of the horse serum, for the material composing the edema must necessarily come from the blood and lymph capillaries in which this antigen is circulating and whose permeability has

been increased by the inflammatory process. A reaction between the sensitized inflamed tissue cells and the antigen could now take place even though the concentration of the horse serum were not greater in the inflamed and edematous area than in the lymph spaces of non-inflamed tissues of the same animal. The reason is that in the inflamed tissues the metabolism of the cells is greatly increased, and we may therefore justly assume that a larger amount of antigen-bearing material affects the inflamed sensitized cell per unit of time than affects the non-inflamed cell in the same interval. Thus the stimulus of a certain concentration of horse serum which is subliminal for a non-inflamed sensitized cell, may rise above the threshold value when an inflamed sensitized tissue is concerned, and an anaphylactic reaction occurs.

On the basis of this interplay of known conditions, the lesions observed in the sensitized and reinjected rabbits are easily explained as the expression of a primary, local, anaphylactic reaction in the skin and subcutaneous tissues of the xylol-treated ears.

Since the explanation and working hypothesis demand the interaction of antigen and sensitized cells of the skin, the phenomenon of Arthus[7] suggests itself at once as a well known example of this type of reaction, and I believe that the ear lesions described above are a variation of Arthus' phenomenon. There are, however, fundamental differences between the procedure resulting in the Arthus reaction and that causing the ear lesions described in this paper.

The Arthus reaction is a skin reaction only, and results when a repeatedly injected rabbit receives a final dose of antigen subcutaneously, the site of the last injection then developing the lesion, the severity of which depends upon the degree of sensitization. In the procedure described in this paper the sensitized animal reinjects itself where there are inflammation and edema, provided that some of the antigen is circulating, the amount circulating being so dilute that it is ineffective under ordinary conditions. Another fundamental

[7] Arthus, M., *Arch. internat. physiol.*, 1908–09, vii, 471. In this article the three notes originally published in the *Compt. rend. Soc. biol.*, 1903 and 1906 (Arthus, M., *Compt. rend. Soc. biol.*, 1903, lv, 817; Arthus, M., and Breton, M., *Compt. rend. Soc. biol.*, 1903, lv, 1478; Arthus, M., *Compt. rend. Soc. biol.*, 1906, lx, 1143), are reprinted.

difference is that the site of reaction, according to this conception, is not always the skin, as in Arthus' phenomenon, but may be any tissue capable of exhibiting an anaphylactic reaction. The reaction, therefore, may take place in the skin, the bronchial muscles, the gastrointestinal canal, the heart, the pulmonary artery, and the nerves, which are structures in which anaphylactic reactions have been described.

That the skin was chosen in the present research was a mere matter of convenience. It was selected because the changes could be directly observed at any time, thus avoiding the complication and difficulties due to indirect observation which always demands instrumental aid. As a matter of fact, preliminary series of experiments had been made on the kidney and on the intestine to test the working hypothesis, but they were abandoned because for the time being it was impossible to carry out the necessary routine work in series of animals sufficiently large to warrant the trial.

It is possible that this process of autoinoculation will aid in explaining the causation of a number of more or less temporary functional derangements of obscure origin in the human subject. In my opinion it is quite probable that sensitization to one or several alien proteins exists much more frequently in man than is suspected, the undenatured protein gaining access to the circulating juices by way of a permeable gastrointestinal mucosa or the nasal and pharyngeal mucous membranes by inhalation; the uncritical use of sera and vaccines at the present time also undoubtedly adds to the number of those sensitized unnecessarily. Severe intoxication of the sensitized individual, however, occurs but rarely since the amount of foreign protein necessary to cause obvious general symptoms on reinjection exceeds hundreds and thousands of times the amount which sensitizes. It is in such individuals that the mechanism described in this paper may come into action and produce a large variety of effects.

It is fully realized that the working hypothesis here developed at some length is not absolutely proved by the experimental data and that much more work is necessary to establish it definitely. It is believed, however, that the experimental results support the conception and that this view renders some functional abnormalities in the human subject more accessible to our understanding.

SUMMARY.

The skin irritant, xylol, when applied to the ears of sensitized and reinjected rabbits often causes a severe inflammation with the formation of crusts and destruction of tissue. Dry gangrene of the entire ear tip may result (Figs. 1, 2, and 3).

The same agent, applied in the same dosage and in the same way, to the ears of (1) normal rabbits, (2) normal rabbits injected once with horse serum (serum controls), or (3) sensitized but not reinjected rabbits, causes only a mild inflammation with more or less edema. The inflammation and edema disappear in 2 or 3 days, leaving a practically normal ear.

The ear lesions of the sensitized reinjected rabbits which develop after the application of xylol are interpreted as a primary anaphylactic reaction. This primary anaphylactic reaction is considered the result of a local autoinoculation of the ear tissues with circulating antigen. The local autoinoculation is brought about by the irritant action of the xylol which causes an inflammation and edema of the site of application. An anaphylactic reaction may now occur because the inflamed tissues are more active metabolically than normal tissues and therefore the inflamed cells are affected by more antigen per unit of time than the normal cells. A subliminal concentration of antigen for non-inflamed, sensitized cells may thus become effective when inflamed sensitized cells are concerned.

This process may theoretically occur in any tissue of a sensitized animal which can show an anaphylactic reaction, for example the intestines, lungs, heart, skin, nerves, arteries, etc. It is possible that this interplay of conditions may explain a number of functional abnormalities in the human subject.

EXPLANATION OF PLATES.

The three figures were made from a rabbit of Series 3. Sensitization, reinjection, and application of 1 cc. of commercial xylol to the left ear were carried out as described in the text.

Plate 45.

Fig. 1. 12 days after the application of xylol; internal surface. The figure shows well the curling of the ear tip, which was dry and hard, the occurrence of hemorrhages near the gangrenous part, and the appearance of discrete blisters.

Fig. 2. 20 days after the application of xylol; dorsal surface. The dry, gangrenous tip has been removed, leaving a thickened, nodular, irregular, bluish border. Some of the crusts on the dorsal surface have also been removed to show the extensive destruction of the skin.

The dorsal surface of the right ear shows a central area where a moderate dermatitis with crust formation took place; this was due to contact of the right ear with the left shortly after application of xylol to the latter. The same effect was observed repeatedly in the sensitized reinjected groups, but not among the controls.

Plate 46.

Fig. 3. 30 days after the application of xylol; dorsal surface. The ear has healed practically completely; note the thickened borders. The ear is largely bald but near the root some white hair is beginning to appear. This white hair gradually covered the ear stump and has remained white now for 5 months.

The right ear patch has also healed completely and shows a few white hairs. 5 months later the hair was still white.

Fig. 1.

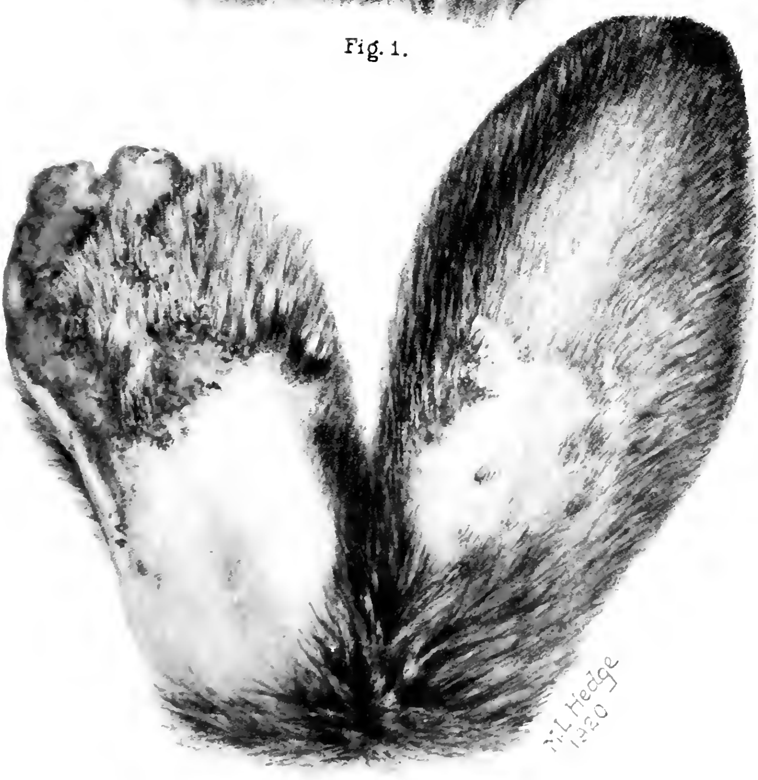


Fig. 2.

(Auer: Local autoinoculation.)

FIG. 3.

(Auer: Local autoinoculation.)

EXPERIMENTAL SYPHILIS IN THE RABBIT.

IV. Cutaneous Syphilis.

Part 1. Affections of the Skin and Appendages.

By WADE H. BROWN, M.D., and LOUISE PEARCE, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 47 to 58.

(Received for publication, June 14, 1920.)

In the preceding papers of this series (1–4), experimental syphilis in the rabbit has been presented from the standpoint of the phenomena of the infection which develops about the site of the inoculation. Ordinarily, these are the only manifestations of infection which are observed in the rabbit, but under certain circumstances, a generalized disease may be produced which is analogous in many respects to that of man, and in taking up the generalized infection, it will be necessary to give a brief résumé of the development of this phase of the subject in order that our work may appear in the proper relation to that of other investigators.

When it had been clearly established that the syphilitic infection could be transmitted to rabbits and maintained with undiminished virulence through successive generations of transfers, interest began to be centered upon problems of a generalized infection. From the outset, opinion was divided as to whether the infection in the rabbit became generalized as in man or remained essentially a localized infection. This division of opinion was in part an outgrowth of the controversy in regard to generalized infections in the lower monkeys and in part a result of experimental observation.

At all events, the development of lesions at points remote from the site of inoculation appeared to be an exceptional occurrence. The first instance of an infection of this kind was reported by Grouven in a series of communications beginning in 1907 (5–8). This case has become classic, and it is worthy of note that it still is the most pronounced example of a generalized infection following local inoculation which has been recorded in the literature. In fact, with a few exceptions, this animal exhibited all the manifestations which have since been

recognized as characteristic of generalized syphilis in the rabbit. In brief, the conditions noted included weakness, emaciation, dyspnea, alopecia, papular and maculopapular lesions of the skin, infiltrations and rhagades about the anterior nares associated with a mucopurulent discharge, conjunctivitis, a metastatic keratitis, and an infection of the testicles, epididymis, and regional lymph nodes, all developing from a unilateral infection of the eye and subsequent enucleation of the infected organ. The first manifestations of a generalized infection appeared 8 months after inoculation and active lesions were still present when the animal died 9 months later.

These observations of Grouven stimulated a great deal of discussion concerning generalized syphilis in the rabbit. Grouven himself reported two other cases (8), and in 1909, a fourth case consisting of a double keratitis originating from a testicular inoculation was reported by Menzincescu (9). About the same time, Uhlenhuth and Mulzer (10) reported a case of metastatic involvement of the scrotum of an uninoculated testicle with papular lesions about the anus, and Truffi (11) reported two cases of keratitis in animals inoculated in the scrotum. Subsequently, other cases of generalized infection were added to this list, but the total number of cases recorded in the literature has remained comparatively small, and most of the animals have shown only a few lesions of a minor character.

In addition to occasional instances of animals showing generalized lesions, there were other facts, however, which indicated that the infecting organism itself was not confined to the region of inoculation. As has been mentioned, Ossola (12) and Truffi (11) demonstrated the presence of spirochetes in the inguinal lymph nodes of rabbits inoculated in the scrotum in 1909, and as early as 1908, Neisser (13) reported successful inoculations of monkeys with material from the spleen and bone marrow of three out of seven rabbits killed 7 to 8 weeks after inoculation with a spleen-bone marrow emulsion from monkeys. These rabbits had been inoculated in the testicles, and although no local lesions had been detected up to the time they were killed, it was claimed that the virus was recovered from the internal organs. As reported by Neisser, these experiments might not be regarded as conclusive, since the only evidence of a specific infection submitted was the production of lesions in the inoculated monkeys which were said by Siebert to be typical primary lesions. At first, other investigators had difficulty in verifying these results, but eventually Truffi (14) succeeded in one instance (number of attempts not stated) in producing a definite infection by inoculation of bone marrow from an animal infected in the scrotum, and similar results have since been obtained by other investigators, notably by Uhlenhuth and Mulzer (15). The obvious objection which might be raised to these experiments, however, is that the results obtained appear to have been inconstant, and no one could say what proportion of the animals would have developed generalized lesions had they been permitted to live.

The occasional case of an animal with manifestations of a generalized infection or the recovery of the virus from the blood and internal organs of even a small

proportion of infected animals was sufficient, however, to establish the fact that generalization of the virus did occur in some instances, but no large series of experiments was carried out, so far as we are aware, for the purpose of determining the time and frequency or the extent of the generalization which took place, and this type of information was much needed to place the question of a generalized infection upon a proper basis.

Further than this, there has been little effort to correlate the phenomena of generalization and the production of generalized lesions. All that was determined was that some animals did show a dissemination of the virus, but in the experience of most observers the appearance of lesions other than those at the site of inoculation was the exception rather than the rule. For example, out of some hundreds of rabbits studied by Uhlenhuth and Mulzer (15), there were comparatively few which showed generalized lesions other than a metastatic infection from one testicle to the other or an occasional case of keratitis, and again Baeslack in 1916 (16) reported that there were no cases of generalized lesions in about 800 rabbits which he had studied.

On the other hand, a few investigators have observed generalized infections with greater frequency. In 1914 Nichols (17) reported the occurrence of generalized lesions of various types in one-half of a series of animals inoculated with a nervous strain of *Treponema pallidum*. Among the conditions mentioned were papular lesions of the eyelids, metastatic lesions of the scrotum and testicles, and lesions of the eyes. In this last group, there was included a new form of lesion of the eye ground recognizable by ophthalmoscopic examination. Unfortunately, neither the relative nor the absolute frequency of the various types of lesions was given, but the investigations were continued by Reasoner (18) who mentions among the manifestations produced by this organism "fundus involvement in about 75 per cent of the rabbits, extension to the opposite testicle in 10 per cent of cases, keratitis occasionally, involvement of the nasal mucosa frequently, with the presence of organism in the nasal discharge. Periostitis of the nasal bones is a later manifestation. There are eyelid lesions in 5 per cent. This strain also may cause mucous lesions of the penis and sheath. It occasionally causes a paronychia, and often a moderate degree of alopecia." But here again, Reasoner frequently refers to the use of repeated and combined testicular and intravenous inoculations, and it is not clear whether the facts recorded above represented results of testicular inoculation alone or included also results obtained from mixed and repeated inoculations of various kinds. In his conclusions, he states that "ordinarily the rabbit develops only an initial lesion, following inoculation in the genitalia, eyes and eyebrows."

On the whole, therefore, our knowledge of generalized syphilis in the rabbit resulting from local inoculation has rested to a large extent upon a proven dissemination of the infecting organisms, the time, frequency, and extent of which were unknown, and the occasional development of a few lesions at some point more or less removed from the site of inoculation, but no comprehensive description has been given of the infection thus produced.

Uhlenhuth and Mulzer (19, 20) described a generalized infection in the rabbit produced by a distinctly different mode of procedure, but even here the picture presented is incomplete. Since these investigators were unable to obtain a severe generalized infection from a local inoculation, they attempted to produce such a condition by resorting to a generalized inoculation, using intracardial and intravenous injections of very large doses of organisms. In this way, they succeeded in producing with considerable frequency what were in reality multiple primary lesions, first in young rabbits and then in adult animals. Considering the mode of inoculation employed, these infections were, of course, more analogous to intrauterine than to postnatal infections. Clinically, they were characterized by emaciation and weakness, especially in young rabbits, associated with lesions of the skin and appendages, the mucous membranes, the genitalia, the eyes, and tumor-like masses about the nose and tail. The extent of the involvement as well as the number and character of the lesions present varied considerably in different animals; there was an especial tendency to exudative affections of the mucous membranes, and the nose and tail tumors together with affections of the nails were the chief additions to the list of syphilitic manifestations which had been previously described. In other words, the lesions produced in these animals were of much the same character as those which had been reported in cases resulting from local inoculation but were, on the whole, more marked and could be produced with much greater frequency.

These experiments appear to have been accepted as a basis for constructing the picture of generalized syphilis in the rabbit and lent considerable support to the contention that marked generalized infection from local inoculation did not occur except in an occasional animal. Of course, the fallacy in this line of reasoning lay in the apparent assumption that a widely disseminated primary infection such as that produced by Uhlenhuth and Mulzer was comparable to conditions which obtained in the secondary diffusion of organisms from an established focus of infection. This error of conception, as will be seen, was a very serious one and had the effect of diverting attention from fundamental problems of the infection.

Since the publication of the work of Uhlenhuth and Mulzer, interest in experimental syphilis has abated somewhat and the only contributions of note which have appeared during the past few years are those of Nichols and of Reasoner.

While these earlier observations were doubtless correct as far as they went, the impressions created were founded upon an imperfect understanding of the experimental infection; they not only left unsettled many questions relative to the generalized infection but gave an erroneous impression as to the nature of the infection produced by local inoculation.

We have been able to collect a large amount of material bearing upon this phase of experimental syphilis which will be presented in

the following series of papers dealing with clinical manifestations of generalized syphilis in the rabbit. We regret that the pathology of these conditions cannot be presented in connection with their clinical history. We have, however, an abundance of material and hope that eventually we may be able to return to this phase of the subject and supply the pathological connection.

Source and Nature of the Material Studied.

The material which forms the basis for our description of generalized syphilis in the rabbit is composed of two groups of cases. Up to September 1, 1919, we had been able to collect 126 rabbits with outspoken manifestations of generalized syphilis developing from local inoculations in the testicles or scrotum. This list of animals included only those in which visible or palpable lesions were present at points entirely removed from the site of inoculation. Thus, metastatic lesions of the testicles and scrotum were not included among the generalized affections on account of the possible confusion of true disseminated lesions with extensions or transformations of processes arising from the primary focus of infection.

This first series of animals came from stock transfers and routine experiments connected with the use of the experimental infection in the rabbit as a means of studying the action of drugs against syphilitic infections and was composed of three classes of animals: (1) those in which the infection ran an undisturbed course; (2) those in which the generalized lesions developed subsequent to castration or excision of primary lesions; and (3) those in which the lesions followed drug administration.

From observations made in the course of routine work, together with an analysis of this group of cases, it appeared that the entire problem of animal resistance to *pallidum* infections, and hence the occurrence of generalized lesions, was intimately connected with the nature and extent of the reaction which took place at the site of inoculation, and that any influence which was capable of modifying this reaction might be expected to react upon the phenomena of the infection as a whole. In particular, it appeared that any condition which tended to lessen, to restrain, to inhibit, or to suppress this reaction without exerting a comparable effect upon the organisms them-

selves might be expected to favor the production of generalized manifestations of disease.

Accordingly, a series of experiments was carried out, aimed primarily at the fundamental problem of the nature of the animal infection and the mechanism of animal resistance. Thus, it was found that such simple means as a unilateral instead of a bilateral inoculation, unilateral or bilateral castration, or the use of therapeutic agents capable of inducing resolution of the primary lesions without destroying the infecting organisms, would completely alter the character of the infection and lead to the production of generalized lesions in a very large proportion of the animals inoculated.

From these experiments, a second group of animals with generalized syphilis has been collected which places the total number of cases available for study at well over 200.

The study of these animals was chiefly clinical. In the majority of cases, the lesions were allowed to pursue an uninterrupted course; in individual instances, they were excised for histological study, and some animals were killed at various periods of the infection in order to make a more thorough examination of existing conditions. The period of observation varied very considerably. Under the circumstances of our work, it was impracticable to hold most of these rabbits for any great period of time. The length of observation in most instances varied from a few weeks to several months; some animals were held for a year to 18 months and a few for more than 2 years.

The identification of syphilitic lesions rested upon the general character and clinical course of the lesions, the demonstration of spirochetes, and histological examination. In a few instances, therapeutic tests were used in the study of conditions of doubtful or uncertain character and as confirmatory diagnostic measures.

The manifestations of disease which we were able to recognize during the life of the animal fell into the five following groups and will be considered in this order: (1) affections of the skin and appendages; (2) affections of the mucous membranes and mucocutaneous borders; (3) affections of tendons, tendon sheaths, periosteum, cartilage, and bone; (4) affections of the eyes; and (5) lymphadenitis. In addition, certain visceral lesions, notably of the heart and of the central nervous system, were discovered at autopsy. This group of conditions will be reported later.

In presenting this material, we shall confine ourselves for the present to a general presentation of the subject, since it has been found that the incidence of different groups of affections, the character of the lesions present in a given case, their time of occurrence, and the general course of the disease are all more or less influenced by the circumstances under which the generalized disease makes its appearance.

Lesions of the Skin and Appendages.

The first group of conditions to be considered are the affections of the skin and appendages including alopecia, paronychia, onychia, and lesions of the skin proper. Affections of this class occurred in a large proportion of the animals showing manifestations of generalized syphilis, and it will be necessary to divide the subject matter of these conditions into two papers the first of which will be confined to a description of the individual affections, while the second will deal with the clinical aspects of cutaneous syphilis.

Alopecia.

Local or general roughening of the coat and falling out of the hair are among the conditions most frequently mentioned as manifestations of generalized syphilis in the rabbit and are extremely common among rabbits infected with *Treponema pallidum*, but occur also from many causes in uninfected animals, the chief ones being moulting, diseases of the skin, systemic disease, and uncleanly habits. While we are reasonably certain, therefore, that a large number of our rabbits showed abnormalities of the coat referable to their syphilitic infection, we realize that it is very difficult to identify such cases with absolute certainty. In studying these affections, we first attempted to exclude the four causes mentioned, which can be done without much difficulty in all except the moulting, or shedding, of the animal. It was found that under laboratory conditions, rabbits were very irregular in this respect and might shed their coats at almost any time from early March to late November, which left only about 3 months of the year during which one was not constantly confronted by this possibility. As far as we were able to determine, there was no pathognomonic sign of syphilitic alopecia in the rabbit. Among the animals studied, there were comparatively few in which we felt justified in making such a diagnosis, and in nearly all instances the diagnosis was supported by the presence of other lesions whose character could be established with absolute certainty.

As nearly as could be determined, there appeared to be at least three conditions which might be regarded as syphilitic alopecia. The most common of these

was one in which the coat became roughened or unkempt; the hair was dry and without luster and was continually falling out. This condition might affect the entire coat, as in Fig. 1, or only some smaller area, and was especially common about the head and ears, giving to these parts a characteristic moth-eaten appearance (Fig. 2). This form of affection is probably the one usually referred to as syphilitic alopecia in the rabbit.

No true baldness was noted in affections of this kind. In exceptional instances of diffuse alopecia, there was decided thinning of the hair over certain areas of the body such as the thighs, the thorax, the abdomen, or about the elbows (Fig. 3). Facial alopecia, alopecia of the ears, and of the cheeks were more marked as a rule, and the hair frequently came away from these areas in considerable masses (Figs. 3 and 4) or with gentle rubbing patches were left which were entirely denuded of hair.

A second form of alopecia which might be referred to a syphilitic infection was characterized by no other manifestation than looseness of the hair. The coats of these animals appeared to be in perfect condition but upon gently plucking at the hair, it was found to be so loose as to come away in handfuls, leaving the skin perfectly bare or covered with a short stubby growth of hair as in Figs. 5 and 6. This condition was so marked in some animals that it was possible to pluck the fur from large areas of the body without inflicting the slightest traumatism to the skin. In some instances, the skin itself appeared entirely normal, while in others, removal of the hair revealed the presence of unsuspected lesions (Fig. 5). A similar condition was found to occur in some animals at the time of shedding. In these cases, however, the growth of new hair was nearly always well advanced before the old hair could be removed, which was not the case with the supposedly syphilitic affection.

Both these forms of alopecia were only temporary affections as a rule, and after a few weeks or months, the coat returned to a normal condition. They appeared to be intensified, however, at the shedding periods of normal rabbits, which in itself is not surprising. In one animal of our series, which was held under observation for about 18 months, there was no time at which the entire region over the hips, thighs, and loins could not be picked entirely free of hair, and as fast as the hair returned, it could be plucked out again with but the slightest tension. A similar condition existed over other portions of the body, but otherwise the coat appeared to be in remarkably good condition, as may be seen by reference to Fig. 6.

A third form of alopecia which bordered upon a true baldness was seen in a few animals. This condition was usually confined to an area a few centimeters in diameter and was characterized by what might be called a peeling of the fur, the roots of which were matted together by superficial layers of the epidermis. By rubbing these areas, masses of fur came away, leaving a bare skin covered with fine epithelial scales. The skin showed a variable degree of thickening, and in some instances focalized infiltrations and necrosis as well. A typical

though rather pronounced case of this kind is illustrated in Fig. 8. The condition here shown extended from the cheeks down over the neck and shoulders where there were two well defined areas of infiltration and necrosis of the skin.

With recovery of these lesions, there was a thin stubby growth of hair but no return to normal as in the preceding cases. This condition might be classed as a true skin lesion rather than as an alopecia, but the alteration in the skin was so variable and in some instances so slight that the alopecia appeared to be the most characteristic feature of the condition.

There was one other condition noted which may be mentioned, the etiology of which could not be determined with certainty. It affected the hair of the face and especially that over the bridge of the nose, and was characterized by a thinning of the hair over the affected area while that remaining appeared broken and irregular much as in the case of tinea infections (Fig. 7). The skin of the affected part showed a slight infiltration in some cases, but in others was thin or atrophic. The only causative factor which suggested itself besides that of a specific infection was the rubbing of the animal against the cage, and this seemed unlikely. Once the condition developed, there was little or no tendency toward a return to normal.

Onychia and Paronychia.

Onychia and paronychia have been mentioned among the manifestations of generalized syphilis in the rabbit, but in our experience, affections of the nails which could be definitely ascribed to syphilitic infection were comparatively rare—only seven cases having been recorded among 126 rabbits and only one of these could be regarded as a true onychia.

Paronychia was first recognized by a slight reddening and swelling of the skin about the base of the nails. The skin then became thickened or infiltrated and was covered with yellow or yellowish gray scales or crusts producing a condition like that shown in Figs. 9, 10, and 12. In extreme cases, the reaction about the base of the nail was much more marked and resulted in the formation of granulomatous masses which underwent secondary necrosis and ulceration with consequent disturbance of the nutrition of the nails themselves (Figs. 11 and 12).

These conditions were usually bilateral and symmetrical and affected the nails of both the front and hind feet.

Syphilitic paronychia was found to be difficult to distinguish in some instances from a non-syphilitic affection of a similar character which is comparatively common among rabbits. This fungus or parasitic disease of the nails develops sooner or later in all rabbits with fungus or parasitic infections of the skin or external ear. While

these affections occur upon the hind feet as well as the front, they usually make their appearance about the toes on the median side of the front feet and are not confined to the region of the nails but spread diffusely over the interdigital surfaces. In contrast to this, syphilitic paronychia appeared to be more common about the nails of the lateral toes and was sharply confined to the base of the nail. Again, the parasitic disease is a steadily progressive affection and never clears up spontaneously, while the syphilitic condition is variable in its course and clears up completely without any treatment. If spirochetes can be found, they are helpful in making a diagnosis, but in their absence, one has to rely upon clinical characteristics.

A true onychia, as has been mentioned, was recognized by naked eye observation in only one of our first group of rabbits. In this case, the condition was associated with a marked paronychia of some of the toes, and the alterations in the nails were so pronounced as to leave no doubt as to the etiological factor concerned (Fig. 11). A similar but less marked condition was also presented by the animal shown in Fig. 12. In other instances it was noted that the nails showed signs of wearing short or appeared roughened and broken towards their ends and tended to split and scale just as in the case of the outer and inner toes of the animal in Fig. 11. An example of nail involvement of this type with no associated paronchyia is shown in Fig. 13.

At the time these observations were made, no relation could be established between this condition and the syphilitic infection. Subsequently, however, it was learned that alterations such as these might arise from involvement of the nail bed. In the rabbit, the nail fits closely over the terminal phalanx, and periostitis with considerable destruction of these bones may take place without giving rise to any external evidence of the existence of such an infection other than an alteration of the nails of the affected toes.[1] Onychia in the rabbit appears, therefore, to be most often associated with a periostitis and is difficult of recognition by the use of ordinary means of diagnosis. If the involvement is slight, the nail may eventually recover, but if the destruction is extensive, the effect appears to be permanent or at least of long duration.

[1] These observations were made by Dr. W. H. Brown, Dr. L. Pearce, and Dr. W. D. Witherbee in the course of a series of investigations of deep seated bony changes by the use of the x-ray, the results of which will be reported later.

Cutaneous Lesions.

The cutaneous lesions formed a very large and varied group of affections. They included lesions of the macular, papular, and nodular or tubercular varieties, and while they possessed many features in common with the cutaneous lesions of man, they differed from them in so many respects that it would be difficult to attempt a complete correlation of the two classes of lesions upon the basis of the material which is at present available. The only classification of cutaneous lesions of the rabbit which seems justifiable at this time is one based upon very broad lines of differentiation such as that afforded by developmental or pathological characteristics.

As was pointed out in connection with scrotal infections, one of the most striking features of the skin reaction of the rabbit to localized infections of *Treponema pallidum* is the tendency to proliferation on the part of the fixed tissue cells and the formation of large granulomatous lesions; a second characteristic of the skin reaction is the tendency to a more or less diffuse infiltration associated with varying degrees of desquamation of surface epithelium, exfoliation, and surface erosion, or necrosis and ulceration; while a third feature of the reaction is a localized hyperemia or even hemorrhage which is associated with varying degrees of exudation expressed chiefly in the form of an edema.

These characteristics of the primary reaction in the scrotum find their counterpart in the reaction to localized infection in other skin areas and form, therefore, an acceptable basis for a consideration of the cutaneous lesions of generalized syphilis. Upon this basis, cutaneous lesions will fall into three classes, the hyperemias, the infiltrations, and the granulomata. It should be understood, however, that there is no sharp line of distinction between these three classes of conditions as there is always a tendency to a combination of the three processes, and such distinctions as can be made must be based upon the predominance of one or another of the three forms of reaction.

In taking up the discussion of cutaneous lesions, the above sequence will be reversed in order to begin with those conditions which are the most obvious; namely, the granulomata.

Cutaneous Granulomata.

The lesions classed as cutaneous granulomata (Figs. 14 to 20 and 22) included those affections which developed in consequence of a reaction which was marked by proliferation of fixed tissue cells and the formation of circumscribed elevated nodules of a fleshy character varying from a few millimeters to several centimeters in diameter. Lesions of this type were quite common. They usually occurred singly or in small groups and as a rule were few in number, but occasionally they were fairly numerous and as many as twenty-eight lesions have been counted in a single animal at one time.

The granulomata appeared either in the form of rather diffuse areas of thickening which involved a considerable depth of the skin or as sharply circumscribed and indurated nodules (Figs. 14 and 17). The more diffuse lesions and the larger nodules were usually of a rose-pink or copper color or were paler than normal and of a faint yellow color, while the small discrete nodules were generally pale and opaque or of a decided opalescent appearance. In exceptional instances, the early lesion was of a deep violet-red color or appeared almost as thickened purpuric spots in the skin. In general, the surface of the lesion was smooth and rather translucent, but during the early stages of its growth there was no marked disturbance of the hair covering its surface. These several conditions are illustrated in the accompanying photographs (Figs. 14 to 19) which show various forms and developmental stages of typical granulomatous lesions.

The evolution of the cutaneous granulomata was usually rapid and within a week or so led to the formation of large oval or irregular spherical masses showing various types of secondary alteration such as are illustrated in Figs. 14 to 23. It is important to note, however, that not all the lesions present in a given case exhibited these changes to an equal degree. As a rule, only a few of those present developed to any considerable extent, while the others underwent involution and completely disappeared. This is a phenomenon worthy of note, since it illustrates the inhibitory influence exerted by one lesion or group of lesions upon another, as was pointed out in connection with the development of multiple scrotal lesions.

The growth of granulomatous lesions took place chiefly from the deeper layers of the skin and tended to be of a concentric type so that surface alterations were usually confined to a relatively small area. The most common changes observed were those due to necrosis. In some instances, this was very superficial and produced no more than a slight exfoliation of epithelium or surface erosion; in others, it extended to a greater depth, and the skin over the center, or even a larger portion of the lesion, became converted into a dry, adherent crust, or a depressed ulcer was formed which converted the lesion into a typical chancre-like mass.

While, as a rule, the area thus involved was small, occasionally a large part of the lesion was destroyed in this manner—the necrosis keeping pace with the growth of the lesion (Figs. 18 and 19).

Another noticeable feature of this class of affections was the preservation of the hair over a large part of the lesion which was obviously due to the nature of the skin involvement. With the advent of infiltration and necrosis of the outer layers of the skin, the hair first became thin and broken and was then lost over the affected area, but, as may be seen by reference to Fig. 19, many lesions showed practically no disturbance of the hair outside the zone of necrosis. In other instances, however, there were a more extensive involvement of the outer layers of skin and an obliteration of papillæ extending well beyond the area of necrosis. In these cases, the loss of hair was more pronounced and lesions were formed which, with their smooth and infiltrated surfaces, bore an even more striking resemblance to the typical scrotal chancre (Fig. 20). Usually the obliteration of skin papillæ and the loss of hair merely preceded the appearance of other changes, but not infrequently they marked the extent of the surface alteration, and the lesions thus formed were quite analogous to unulcerated nodular chancres of the scrotum (Fig. 22).

In many ways, the cutaneous granuloma of the rabbit may be viewed as an expression of a vigorous reaction to infection analogous in all respects to that which characterizes the reaction at the primary focus of infection. Objectively, the force of this statement may be appreciated by comparing Figs. 20 and 21 and Figs. 22 and 23 which represent corresponding cutaneous and scrotal lesions of two animals photographed at the same time. The significance of the striking similarity between these two processes of reaction will be made clear when we come to consider the factors in animal resistance and the mode of expression of this resistance.

Before leaving the subject of cutaneous granulomata, reference may be made to a similar group of lesions which originated within the subcutaneous tissues and reached a considerable size before any skin involvement took place (Fig. 24). These lesions were, of course, more analogous to ordinary gummata, or the so called tertiary skin lesions of man, than to those of earlier stages of the disease, and the few cases seen in the rabbit also appeared late in the course of the infection. For example, the animal shown in Fig. 24 had a succession of cutaneous lesions, the first appearing about 3½ months after inoculation. There were no lesions in the subcutaneous tissues, however, until 25 months after inoculation when the lesion upon the nose developed and was followed 3 months later by other lesions of a similar character.

In all granulomatous lesions, spirochetes were numerous and could be demonstrated without difficulty as long as the lesions were active.

Cutaneous Infiltrations.

The second group of cutaneous lesions to be described (Figs. 25 to 41) includes a variety of conditions ranging from small discrete papules on the one hand to large weeping or crustaceous patches on the other, the common basis of which was a cutaneous infiltration in contradistinction to the proliferative reaction which characterized the lesions of the preceding group. According to available data, these affections occurred among our first series of animals about as frequently as the granulomata, but their incidence has steadily declined with changing conditions until, under present circumstances, they might be regarded as comparatively rare affections.[2] It should be pointed out, however, that lesions of this class were often less clearly defined and much more difficult of detection than the granulomata, and hence it is not unlikely that many may be entirely overlooked.

At the time of their appearance cutaneous infiltrations may differ very slightly if at all from early granulomatous lesions. As in the case of the granulomata, two forms of lesions could be distinguished from their mode of origin, one appearing as a somewhat diffuse process and the other as a very minute and sharply circumscribed nodule, and these characteristics tended to be preserved in the fully developed lesions of this class. There were thus formed two fairly well defined groups of conditions, the one a flattened or lenticular lesion, the other a more elevated and indurated papule.

Flattened Papular Lesions.—The initial lesion of this group (Figs. 25 and 26) presented the appearance of a simple infiltration of the skin, involving the papillary layers and varying from a few millimeters to a centimeter or more in diameter. These spots were usually of a faint pink or copper color, but in exceptional instances, the discoloration of the skin was quite pronounced, approaching in intensity the violet-red color occasionally seen in early granulomatous lesions (Fig. 26). At this stage, the affected area was raised but slightly above the surrounding skin level, the elevation being greatest at the center of the lesion and diminishing towards its periphery.

---

[2] Changing conditions in the life history of the organisms and the particular circumstances under which generalized lesions make their appearance in a given case are undoubtedly potent factors in determining the character which these lesions assume.

Very soon the appearance of the lesions changed, the color becoming a pale yellow or gray or deepening to a copper or brown as the case might be. At the same time, the infiltration increased and the hair over the affected area was lost to a considerable extent, while the skin became smooth and glistening and of a parchment-like consistency or was covered by thin scales of a yellow or yellowish gray color (Fig. 27).

Subsequently, numerous modifications of these lesions occurred as a result of growth or extension or from secondary alterations taking place within the affected area. The characteristic mode of growth of the flattened papular lesion was a peripheral extension of the infiltration which in itself gave rise to a variety of conditions. Most often there was produced a small but fairly well defined area of infiltration analogous to some of those shown in Figs. 28 and 31. Occasionally, however, the process assumed more extensive proportions and from one or more small lesions, there developed a widespread affection such as that seen in Fig. 28. Not infrequently, the original lesions or the center of the lesion in such cases underwent resolution with the peripheral extension of the process as may be seen by an examination of Figs. 28 and 29. There was thus formed a single annular lesion, as in Fig. 29, or an affection composed of a series of lesions grouped in similar fashion about the area in which the infection first appeared (Fig. 28).

In addition to conditions such as those described, numerous modifications of these lesions occurred which were attributable to secondary alterations. Among these may be mentioned a squamous type of lesion, a suggestion of which is given by the accumulation of scales about the margins of the lesion in Fig. 29. Another condition which was of frequent occurrence in this class of affections was exfoliation of the epithelial coverings which gave rise to moist or weeping patches or to areas of infiltration covered by thick yellowish gray or yellowish brown crusts (Figs. 31 and 33). In other instances, necrosis of a more pronounced character occurred and resulted in the formation of an ulcer, as in one of the lesions in Fig. 29, or the entire area of infiltration was converted into a dry, necrotic eschar, a condition which is suggested in Fig. 8.

While in many instances the flattened papular lesions were rather small and few in number, they were occasionally quite numerous and tended to spread and to fuse with one another, as has been described, or were processes of a rather diffuse character from the beginning and covered an area several square centimeters in extent. As might be expected from the nature of the process, this particular type of lesion was especially prone to exfoliation and necrosis and the formation of weeping patches or of lesions of a crustaceous character such as that shown in Fig. 33.

As was pointed out in connection with the cutaneous granulomata, it is important to note again that many of the infiltrative processes which have been described are closely analogous to certain forms of primary scrotal lesions. In order to emphasize this point, two sets of photographs have been inserted to enable one to make the comparison between the primary and the generalized

cutaneous reactions as they appeared in the same animal (compare Figs. 29 and 30, and 31 and 32).

Raised Papular Lesions.—The raised papular lesions of the skin (Figs. 34 to 41) analogous to the miliary or follicular syphilides of man were of three varieties. The simplest of these and the one from which the others usually developed was a small shotty nodule averaging from 1 to 3 mm. in diameter and raised a millimeter or so above the surrounding skin level (Figs. 34 to 36 and 38). These lesions were very sharply demarcated and were of a grayish white or faint copper color. In most instances, they were rather dense and opaque but occasionally were of a semitranslucent or opalescent appearance. The crest of the lesion tended to be rounded or pointed, but in exceptional instances there was a slight umbilication as in Fig. 36. Some of the lesions were also surrounded by a rich vascular network as is clearly shown in this same figure (Fig. 36).

These papules rarely persisted in the condition described for any considerable period of time but tended to undergo one of two types of alteration. Many of them underwent a central or apical necrosis with the formation of a small crust or ulcer. This was particularly the case with lesions situated about the face or ears (Fig. 37). The second modification seen was of a squamous type, and this was especially common with lesions of the upper eyelids and brows (Figs. 38 to 41). In some of these, a thick layer of epithelial scales was formed upon the crest of the lesion (Fig. 40), while in others, the entire nodule was incased in a covering of horny epithelium (Fig. 39). This group of conditions presented the general appearance of small vegetations, and their prominence was due largely to the accumulated epithelial coverings which upon removal left an unexpectedly small and rather flattened body of infiltration.

Another feature of these lesions was the tendency to occur in groups such as those shown in Fig. 39. In two animals of our series, one of which is shown in Fig. 37, the papules showed a definite circinate arrangement and a very striking bilateral symmetry.

Routine examinations for spirochetes were not made upon all the lesions of this group which were encountered, since it was desired to follow the clinical course of the lesions as far as possible, and it was found that the traumatism inflicted either by aspiration with a needle and syringe or by scarification was sufficient to disturb the development of the smaller lesions and predisposed to rapid regression or healing. However, spirochetes were demonstrated in lesions of all the types described. They were most easily demonstrated in the moist or weeping patches of infiltration and in the fleshy papules but were more difficult to demonstrate in the dry necrotic lesions and the small follicular papules. When once familiar with this group of lesions,

however, the clinical history was usually sufficient in itself to enable one to make a perfectly definite diagnosis.

While, in attempting to point out the salient features of various types of cutaneous eruptions, a distinction has been drawn between cutaneous infiltrations and cutaneous granulomata, it must be said that no complete division between these two classes of affections could be made. In reality, they tended to merge one with the other, and while the great majority of the lesions seen in the rabbit could be assigned to one or the other of these two groups, there were borderline conditions which would be very difficult to classify upon this basis.

As our investigations advanced, the difficulties encountered in this respect were increased due to a change in the character of the lesions produced by the two strains of organisms employed. This was especially noticeable in a decrease in the relative incidence of lesions which have been described as infiltrations and a corresponding increase in lesions which clinically might be regarded either as small granulomata or as unusually marked processes of infiltration. Mention is made of this fact on account of the bearing which it may have upon any interpretation which may be placed upon such conditions as manifestations of a reaction to infection.

Macular Erythema, or Roseola.

A macular erythema has never been described among the cutaneous lesions of the rabbit so far as we are aware, but in the course of our own observations, a pronounced rash of this type was seen upon the ears of a number of animals with manifestations of generalized infection. As yet, no conclusive proof of the syphilitic nature of these lesions has been obtained. Thus far, we have been unable to demonstrate spirochetes in the lesions, although we have not had an opportunity of making a thorough search for them in section. The evidence which we have rests upon clinical and histological examinations.

In its typical form, the macular erythema first appeared as slightly thickened, rose-colored spots or patches situated in the deeper layers of the skin or in the subcutaneous tissues of the outer third of the ear. The color of the rash deep-

ened to a dusky red or purple, then changed to a coppery hue, and faded, leaving a faint stain in the tissues. Occasionally, there were small petechial hemorrhages associated with the lesions and a central opaque white spot or ring which persisted after the general color of the rash had faded. A typical though rather pronounced case of macular erythema is shown in Fig. 42.

As a rule, the hair was loosened over the affected area or over a much greater area than appeared to be involved, and, in a few instances, the surface of the skin was also affected, becoming roughened and covered with scales, a condition which was especially noticeable upon the inner surface of the ears where the skin is normally quite smooth. Ordinarily, no other changes were noted, but in two instances, there was a well marked infiltration of the affected areas which made its appearance just as the rash began to fade. The ear of one of these animals and the areas of infiltration are shown in Fig. 43.

In their distribution, the macular lesions usually preserved a fair degree of bilateral symmetry. Aside from the ears, the only positions in which definite purpuric spots of the character described have been seen were the trunk and the thighs of two animals.

During the early stages of the erythema, the color was diminished by pressure or by vascular constriction and increased with dilatation of the vessels, but such influence had little if any effect upon the appearance of the rash after the purpuric stage had been reached. In general, these lesions suggested a reaction similar to the early reaction described in the scrotum following inoculation with a virus emulsion.

This macular erythema was usually fleeting and rarely persisted for more than a few days, and frequently a slight roseola disappeared within 24 to 48 hours after it was first noted. In most instances, the roseola developed early in the course of the infection, but in one animal, it was observed as late as 16 months after inoculation. Another characteristic feature of these lesions was a marked tendency to recurrent periods of eruption.

After a long series of observations, it was found that a very large proportion of the animals which showed eruptions of this type belonged to one of three classes: first, those which had shown other manifestations of generalized infection, second, animals in which such lesions were still present, or third, animals which subsequently did develop lesions of a definite syphilitic character.

Histologically, three types of change were found in these lesions. In the early stages of the erythema, the vessels of the region were

dilated and the surrounding tissues were edematous. At a later period, there was stasis as well as dilatation of the vessels and slight extravasation of red blood cells along with the edema, and the endothelial cells of the vessels were swollen. These were the usual changes, but in the most marked cases, there were in addition a slight migration of leucocytes and an accumulation of round cells. These changes are precisely those which occur with the initiation of the specific reaction in the testicle and scrotum.

The chief source of difficulty in relating the macular erythema to infection with *Treponema pallidum* is the occurrence of erythemata and vasomotor disturbances of various sorts in the ears of normal rabbits. The non-specific condition usually develops from the base of the ears and radiates outward along the marginal vessels but may assume a macular form not unlike that of the early roseola described. These conditions were found to occur especially during periods of moulting, or shedding, and at such times, thickened erythematous patches may be found on any part of the body where new hair is beginning to grow. However, the purpuric type of eruption was never observed except in infected animals, although very many normal rabbits were examined with this point in view.

As the matter stands, therefore, we feel reasonably certain that a true macular lesion does occur in the rabbit, but the difficulties of making a positive diagnosis are so great that we have confined our listing of such conditions to animals which showed some other manifestation of generalized syphilis, and among these, to those animals in which the rash appeared in typical form.[3]

Lesions of Uncertain Etiology.

The cutaneous lesions thus far described include only those affections whose etiology we have been able to establish with absolute certainty or, as in the case of the macular erythema, with a strong degree of probability. This group of manifestations would hardly be complete, however, without mention of several conditions which may

[3] It is possible that therapy exercised some influence upon the occurrence of macular erythemata, since eight of the first thirteen animals in which an outspoken eruption of this character was observed, were drug-treated animals.

bear some relation to *pallidum* infections but whose etiology we have not been able to establish to our own satisfaction.

The first of these conditions is that illustrated by Fig. 44 which shows a bare area of skin in the lumbar region from which the hair has been plucked. In this area, there were two definite nodular masses, one covered by a short growth of hair, the other entirely bare and of a decided red color. A similar reddened and thickened area projects beyond the margins of the upper edge of the denuded skin area. These conditions were comparatively common and especially so at the time of moulting.

It will be noted that the peculiar condition of the skin in this animal is not unlike the early stage of a syphilitic skin lesion but analogous conditions are known to occur among uninfected rabbits. In the presence of a negative examination for spirochetes, obviously no diagnosis of the nature of such affections could be made. Granting, however, that conditions of this kind are entirely phenomena of a new growth of hair, it still appears possible that they may be influenced to some extent by the presence of a syphilitic infection. Whether such is the case cannot be said. The conditions were confined almost entirely to the face, where known skin lesions usually present a very characteristic appearance, and to the trunk, a region in which almost nothing is known of syphilitic lesions. The condition is mentioned mainly to avoid confusion between affections of proven origin and conditions of unknown etiology which may simulate them.

The second group of conditions to which we may refer concerns more especially certain of the hair follicles or possibly the sebaceous glands of the cheeks and, to a lesser extent, the neck. In a considerable number of infected rabbits, it was found that an abnormal condition of the skin developed about the roots of certain hairs, usually late in the course of the disease. The condition was characterized clinically by the formation of an exudate which glued together small tufts of hair irregularly distributed over the cheeks and sides of the neck. This material extended a millimeter or so up the shaft of the hair and resulted in the formation of dry, hard projections which were very easily palpable and were of a cream-yellow or lemon-yellow color. When the hair was clipped, the appearance presented was that shown in Fig. 45.

This condition might persist for a very long time or might clear up after only a short duration. In the process of clearing, the hair came out, and one found beneath these masses of exudate, faint circles in the skin similar to those seen in Fig. 46.

A condition which appeared to be of the same nature developed in several animals whose cheeks had been shaved as a means of investigating suspected skin lesions. One of these animals is shown in Fig. 47. The lesions formed under these conditions were minute points of elevation surmounted by a small crust. It was found that by applying pressure or tension to the skin, a small droplet of clear or slightly turbid fluid exuded from these points. This fluid contained a

large number of polynuclear and mononuclear cells, but no spirochetes could be found.

In this particular animal, it may be of interest to note the existence of another abnormality in regard to the growth of the hair of the area shown. This animal with two others was shaved several weeks before the photograph in Fig. 47 was taken. At that time, there were several small syphilitic lesions at the base of the ears. The hair of the other two animals (both with specific lesions) returned very promptly, but in this animal, there was practically no growth of hair for many weeks which in itself is indicative of disturbed nutrition of the hair, and is a condition which has been repeatedly observed in animals with marked generalized syphilis.

This group of conditions suggests the possibility of a follicular syphilide, possibly a pustular eruption, but at present, it is no more than a suggestion. Similar conditions were found in uninfected rabbits but were far less common and were usually associated with other evidences of abnormality. In infected animals, this condition showed no relation to other diseases. For example, the rabbit in Fig. 45 was in perfect condition except for the presence of two small lesions upon the hind feet. A year previous, however, it had been the subject of a most pronounced cutaneous syphilis.

We are of the opinion that some of these conditions may be directly or indirectly related to infection with *Treponema pallidum*. We have had no opportunity, however, to make a thorough investigation of them and in the absence of any definite proof of a syphilitic origin, they must be left as conditions of uncertain etiology.

SUMMARY.

From the study of a large series of rabbits with outspoken manifestations of generalized syphilis, lesions of the skin and appendages were found to constitute one of the largest and most varied groups of such affections. The conditions noted consisted of alopecias, onychia and paronychia, and lesions of the skin proper.

It was found to be a matter of some difficulty to make a positive diagnosis of syphilitic alopecia, but there were three and possibly four conditions which appeared to be attributable to such an infection. The first of these took the form of a general or local roughening of the coat with falling of the hair which produced the typical moth-eaten appearance associated with syphilitic alopecia in the human subject. A second form of alopecia was essentially an abnormal looseness of the hair which permitted large areas of the body to be completely

denuded. The third type of alopecia was associated with definite skin changes, and the hair was readily removable together with an adherent mass of epithelial scales.

Paronychia was comparatively rare but was readily recognized by a characteristic infiltration and exfoliation of the skin about the base of the nails.

The incidence of onychia is uncertain. Late in the course of the investigation it was found that alterations in the nails which were not entirely characteristic in themselves might occur in consequence of a syphilitic involvement of the nail beds which could not be detected by ordinary methods of examination. The cases which were recognized as syphilitic were those which showed an associated paronychia.

Lesions of the skin were found to be one of the most frequent manifestations of a generalized infection in the rabbit. These lesions were divided into three classes: first, granulomatous lesions, second, infiltrations, and third, erythemata.

The granulomata were lesions of a fleshy character which tended to grow to a very large size and presented all the characteristics of circumscribed primary lesions of the scrotum.

The conditions described as cutaneous infiltrations included two general types of lesions, one a flattened and rather diffuse process, the other an elevated and sharply circumscribed papule. As a class, these lesions were very prone to secondary alterations and in this way gave rise to a great variety of conditions which in general resembled the diffuse primary lesions of the scrotum and the papular lesions resulting from local dissemination.

A third type of lesion resembling the macular erythemata of man was observed in a small number of animals, and while no definite proof of the specific origin of these lesions was obtained, the evidence available was strongly suggestive.

In addition, several other cutaneous affections were noted which have not as yet been thoroughly investigated. It is suggested, however, that these processes may bear some relation to infection with *Treponema pallidum*.

BIBLIOGRAPHY.

1. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1920, xxxi, 475.
2. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1920, xxxi, 709.
3. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1920, xxxi, 729.
4. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1920, xxxi, 749.
5. Grouven, C., *Med. Klin.*, 1907, iii, 774.
6. Grouven, C., *Dermat. Z.*, 1908, xv, 209.
7. Grouven, C., *Verhandl. deutsch. dermat. Ges., X. Kong.*, 1908, x, 212.
8. Grouven, C., *Dermat. Z.*, 1910, xvii, 161.
9. Mezincescu, D., *Deutsch. med. Woch.*, 1909, xxxv, 1188.
10. Uhlenhuth and Mulzer, *Arb. k. Gsndhtsamte,* 1909, xxxiii, 183.
11. Truffi, M., *Centr. Bakt., 1te Abt., Orig.*, 1909, lii, 555.
12. Ossola, *Gior. ital. mal. ven.*, 1909, xliv, 171, cited by Truffi (11).
13. Neisser, A., *Dermat. Z.*, 1908, xv, 73.
14. Truffi, M., *Med. Klin.*, 1910, vi, 269.
15. Uhlenhuth, P., and Mulzer, P., *Arb. k. Gsndhtsamte*, 1913, xliv, 307.
16. Baeslack, F. W., *Urol. Cut. Rev., Techn. Suppl.*, 1916, iv, 15.
17. Nichols, H. J., *J. Exp. Med.*, 1914, xix, 362.
18. Reasoner, *J. Am. Med. Assn.*, 1916, lxvii, 1799, quoted from reprint.
19. Uhlenhuth and Mulzer, P., *Arb. k. Gsndhtsamte*, 1910, xxxiv, 222.
20. Uhlenhuth and Mulzer, *Deutsch. med. Woch.*, 1911, xxxvii, 51.

EXPLANATION OF PLATES.

All the illustrations are from unretouched photographs which, with the exception of Figs. 1 and 6, represent the objects at approximately their natural size.

Figs. 1 to 8. Alopecias in the rabbit. Abnormalities of the coat which may be referable to a syphilitic infection. All the animals here shown had active manifestations of generalized syphilis, other than the alopecia, at the time these photographs were taken with the exception of one in Fig. 6 and in this case there was a double keratitis at the time the alopecia first appeared.

Plate 47.

Fig. 1. An abnormal condition of the coat frequently observed in rabbits infected with *Treponema pallidum.* A diffuse alopecia.

Plate 48.

Fig. 2. Alopecia areata, showing a slightly moth-eaten appearance of the hair about the face and ears. This condition may be simulated by ordinary processes of moulting.

Fig. 3. Diffuse alopecia, showing a marked thinning of the hair over the thigh and a slight roughening of the skin.

PLATE 49.

FIG. 4. Alopecia areata associated with desquamation of surface epithelium, a condition also seen at the time of moulting.

FIG. 5. An animal in which the hair was plucked from the region of the head and shoulders. Elsewhere the hair was firmly set. Note the cutaneous lesions on the face. An affection as pronounced as this is rarely observed in a normal animal but is comparatively common among those infected with *Treponema pallidum*.

PLATE 50.

FIG. 6. An affection of the coat characterized by abnormal looseness of the hair which persisted over a period of more than 18 months. The region over the hips, thighs, and loins of this animal were plucked clean a number of times. Note the excellent appearance of the coat of the animal.

PLATE 51.

FIG. 7. A peculiar thinning of the hair over the lower portion of the nose.

FIG. 8. The same animal as in Fig. 7. Alopecia areata associated with a slight diffuse infiltration of the skin, desquamation of epithelium, and focal necroses. An unquestionable syphilitic affection.

FIGS. 9 to 13. Affections of the nails.

FIGS. 9 and 10. A bilateral paronychia of the front feet.

FIG. 11. Left hind foot. Marked paronychia and onychia of the nails on the two middle toes and onychia of the inner toe. Hair clipped.

FIG. 12. Right hind foot. Paronychia of first and third toes. Onychia with loss of the nail. Hair clipped on the third toe.

FIG. 13. Simple onychia of hind foot. Hair over the toes clipped. This animal showed a periostitis and necrosis of the terminal phalanges.

PLATE 52.

FIGS. 14 to 19. Cutaneous granulomata analogous in many respects to the grouped nodular or tubercular lesions of the so called secondary and tertiary periods of human syphilis.

FIGS. 14 to 16. Stages in the development of a typical cutaneous granuloma as seen at intervals of 1 week. Fig. 14 shows an area of diffuse thickening in the skin, the surface of which was of a decided copper color. In Fig. 15, there is beginning surface necrosis, and Fig. 16 shows a well developed chancre-like lesion with central necrosis and depressed ulcer. Area shaved.

FIG. 17. A group of six early granulomatous lesions on the dorsum and side of the right hind foot with an older lesion over the tendo achillis as seen after removal of the hair. These lesions were all sharply demarcated but varied in appearance from pale opalescent nodules to nodules of a deep violet-red color. Note the tense appearance of the skin and glistening surface of the two largest lesions.

FIG. 18. A group of granulomatous lesions on the right hind foot representing various stages of development. The lesions in this animal were characterized by an intense violet-red color and by rapid and widespread necrosis. The enlargement of the fifth metatarsal seen in the photograph is due to a syphilitic periostitis. Hair removed.

FIG. 19. Granulomatous lesions of the foot illustrating various stages of necrosis and ulceration and the persistence of the hair over this class of lesion. Hair clipped.

PLATE 53.

FIGS. 20 and 21. A cutaneous granuloma and the scrotal lesions of the same animal. The animal was inoculated in the testicles and subsequently the left testicle was removed, but large scrotal lesions developed on both sides, presenting essentially the same appearance as the generalized lesions of the skin.

FIGS. 22 and 23. An unulcerated granuloma of 4 months duration with a bald patch on its surface and the corresponding lesion of the scrotum 11 months after inoculation. The two lesions were photographed at the same time. A similar lesion in the right scrotum had been excised.

FIG. 24. A subcutaneous granuloma, or gumma, freely movable between the skin and the nasal bones, $26\frac{1}{2}$ months after inoculation. Hair clipped.

FIGS. 25 to 41. Cutaneous infiltrations. The lesions in this group represent processes which are perhaps more analogous to the cutaneous lesions of man than those of the preceding group.

PLATE 54.

FIG. 25. Large copper-colored patches of infiltration just appearing on the right ear. Ears shaved.

FIG. 26. A very early but pronounced area of infiltration on the dorsum of the right front foot. The lesion was of a violet-red color and at this time presented much the same appearance as an early granulomatous lesion. The nodular mass at the carpus is a syphilitic lesion of the ulna. Hair removed.

FIG. 27. Small cutaneous infiltrations at the base of the ears showing accumulation of epithelial scales. An early secondary transformation. Area shaved.

FIG. 28. Multiple lesions of the fore arms and feet grouped in irregular circles with the most active lesions towards the periphery. The affection first appeared in the region of the carpus and subsequently extended as shown in the photograph. The lesions were profusely covered by fine epithelial scales most of which were unavoidably lost in shaving the affected area.

PLATE 55.

FIG. 29. The same animal as in Fig. 28. An annular lesion, the margins of which are partly covered with epithelial scales, and a smaller area of infiltration with a necrotic center covered by a scab. A third lesion is seen in profile upon the anterior surface of the ear. Area shaved.

FIG. 30. The primary lesions of the scrotum in the same animal as that in Figs. 28 and 29, intended to show a similarity in the cutaneous reaction of various parts of the body or between the so called primary and secondary lesions of the skin.

FIG. 31. Multiple cutaneous infiltrations on the right front and hind feet showing some loss of hair and various degrees of necrosis and ulceration. The hair has been clipped but not shaved.

FIG. 32. The scrotum of the same animal and the lesions which developed following inoculation. (See legend of Fig. 30.)

FIG. 33. A large crustaceous lesion over the elbow and paronychia of the fifth toe. Natural appearance.

PLATE 56.

FIG. 34. An early papular lesion on the posterior margin of the ear, showing the pale body of the lesion with a narrow zone of color at its base. Area shaved.

FIG. 35. A small papular lesion of a few days duration on the inner surface of the ear.

FIG. 36. Two small papules with central umbilication surrounded by a network of vessels. Lesions of only a few days duration.

FIG. 37. A group of small papular lesions showing circinate arrangement and bilateral symmetry.

PLATE 57.

FIG. 38. A fleshy papule of the upper eyelid.

FIG. 39. Multiple papular lesions of the upper lid and brow covered by heavy epithelial plaques, a transformation which frequently affects lesions such as that in Fig. 38.

FIG. 40. A fungus type of lesion which appears to be due to a continuous piling up of epithelial debris intermingled with a serous exudate. The body of this lesion was hardly more than 2 mm. in diameter.

FIG. 41. The same lesion at a later date showing a very irregular but still somewhat scaly surface. This figure is given to illustrate modifications which may take place in a given lesion.

FIG. 42. A macular erythema, or roseola, of the ear.

FIG. 43. Cutaneous infiltration following the fading of a roseola. There was still a distinct copper color in these areas.

PLATE 58.

FIGS. 44 to 47. Abnormalities of the skin frequently observed in rabbits infected with *Treponema pallidum* whose connection with the *pallidum* infection is still undetermined.

FIG. 44. An area of alopecia in the region of the loin showing irregular patches of thickening in the skin analogous to those which occur with a new growth of hair. These conditions are especially frequent in infected animals.

Fig. 45. Multiple follicular lesions over the cheek and neck. These lesions develop about the roots of certain hairs, and while they are quite common in infected rabbits, they are occasionally seen in apparently normal animals. Hair clipped.

Fig. 46. Alopecia of the cheek and pale circles in the skin occurring with the healing of lesions such as those in Fig. 45.

Fig. 47. A bare area of skin showing several minute crusts with a bristle protruding and numerous irregular areas of thickening and mottling in the skin. The one marked by the arrow was a definite syphilitic papule. The upper part of the area was shaved about 7 weeks before this photograph was taken but there had been practically no growth of hair within this time, which is most unusual.

(Brown and Pearce: Experimental syphilis in the rabbit. IV.)

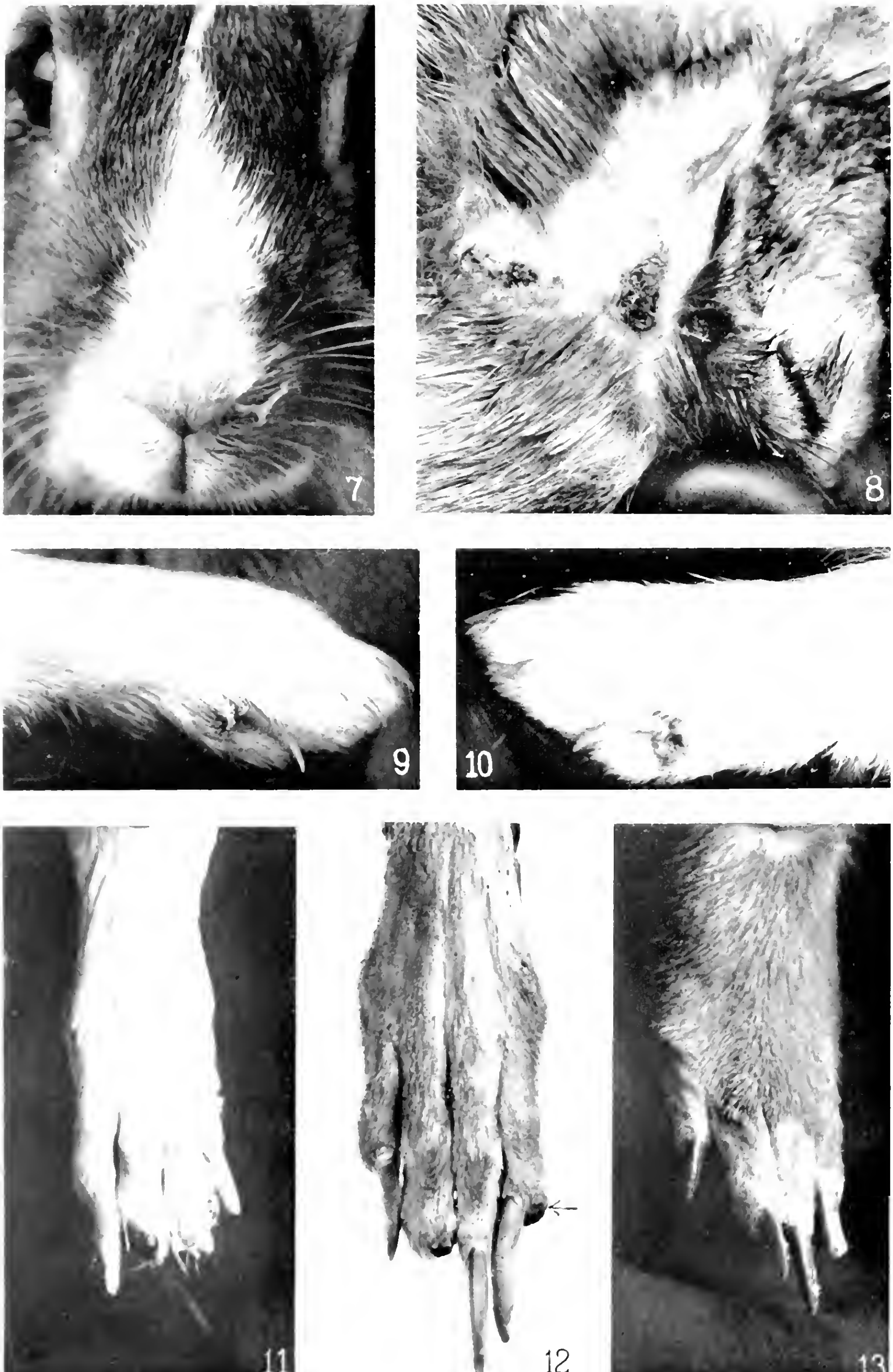
(Brown and Pearce: Experimental syphilis in the rabbit. IV.)

5

4

(Brown and Pearce: Experimental syphilis in the rabbit. IV.)

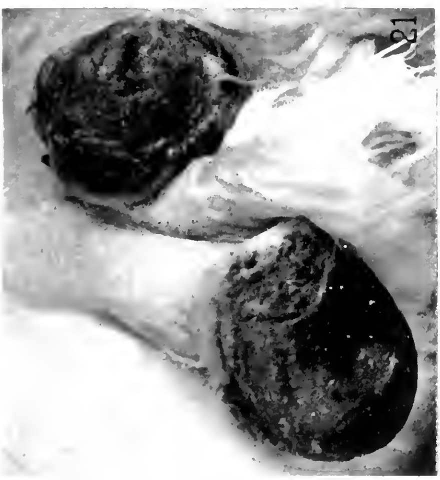
(Brown and Pearce: Experimental syphilis in the rabbit. IV.)



(Brown and Pearce: Experimental syphilis in the rabbit. IV.)

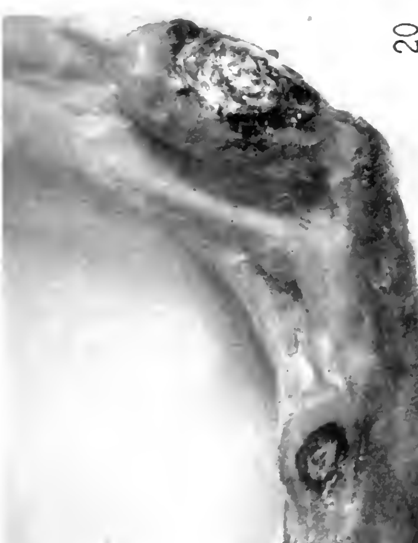
(Brown and Pearce: Experimental syphilis in the rabbit. IV.)

24



21

23



20

22

(Brown and Pearce: Experimental syphilis in the rabbit. IV.)

25

26

27

28

(Brown and Pearce: Experimental syphilis in the rabbit. IV.)


29

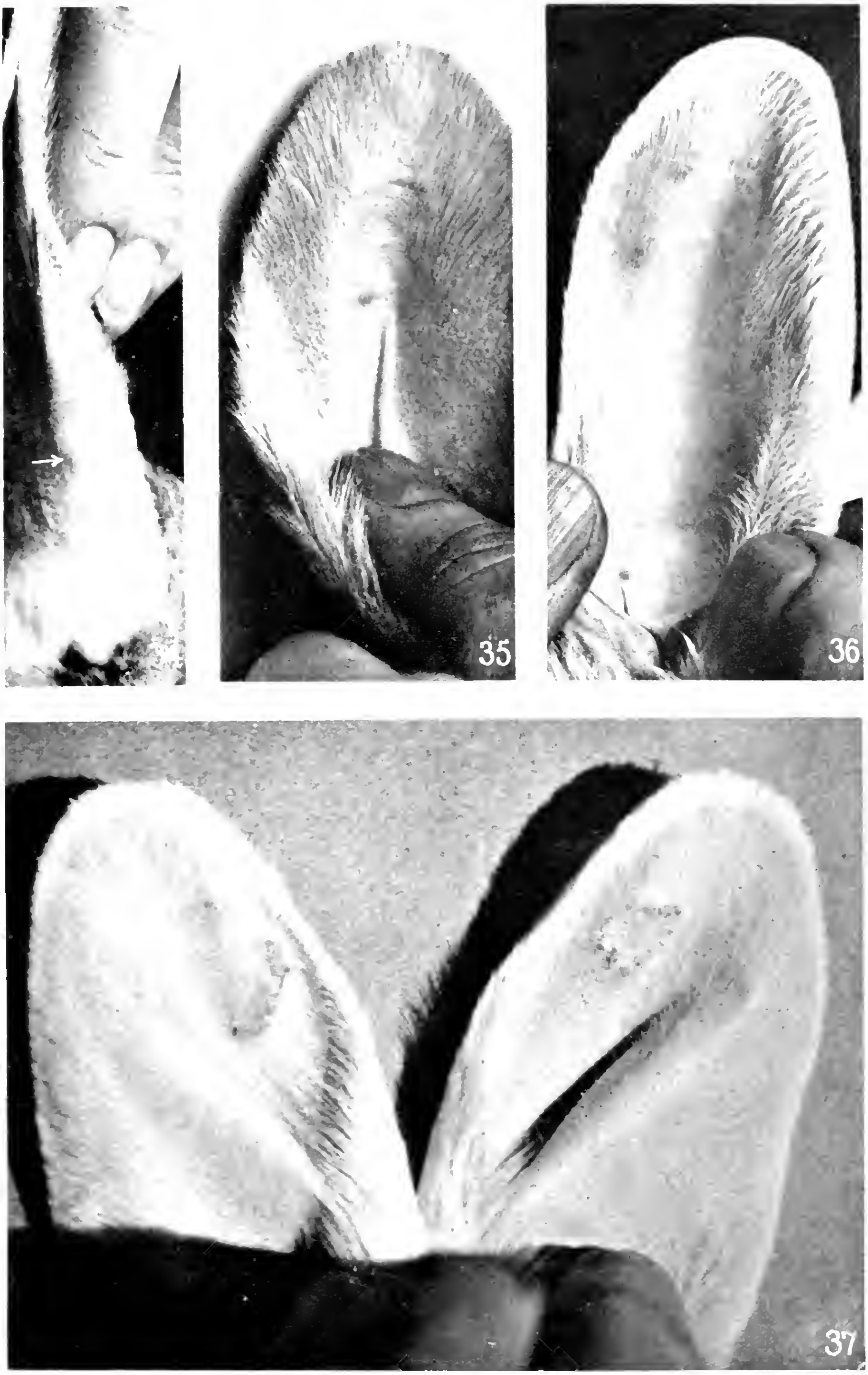

30


31

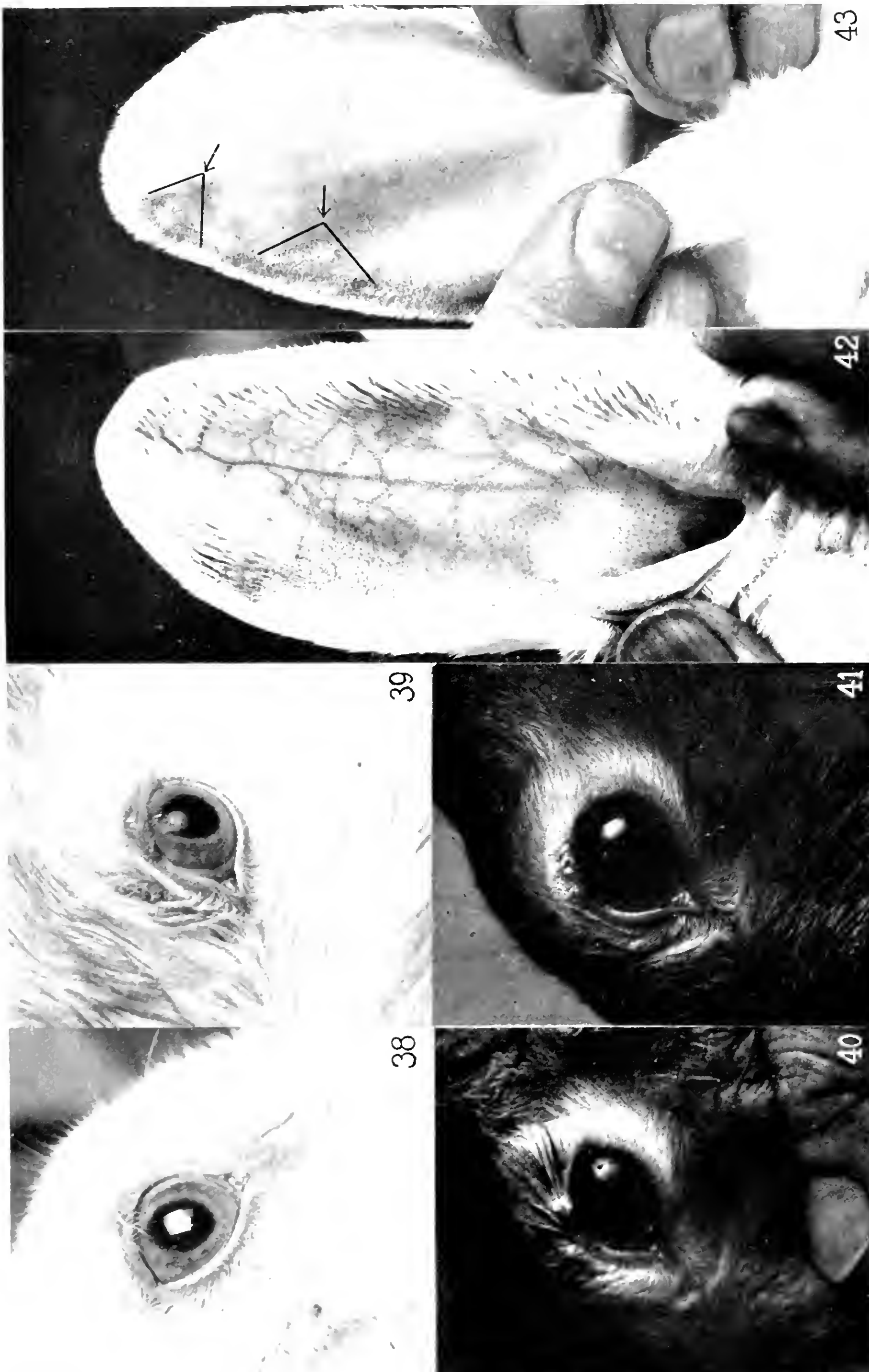

32


33

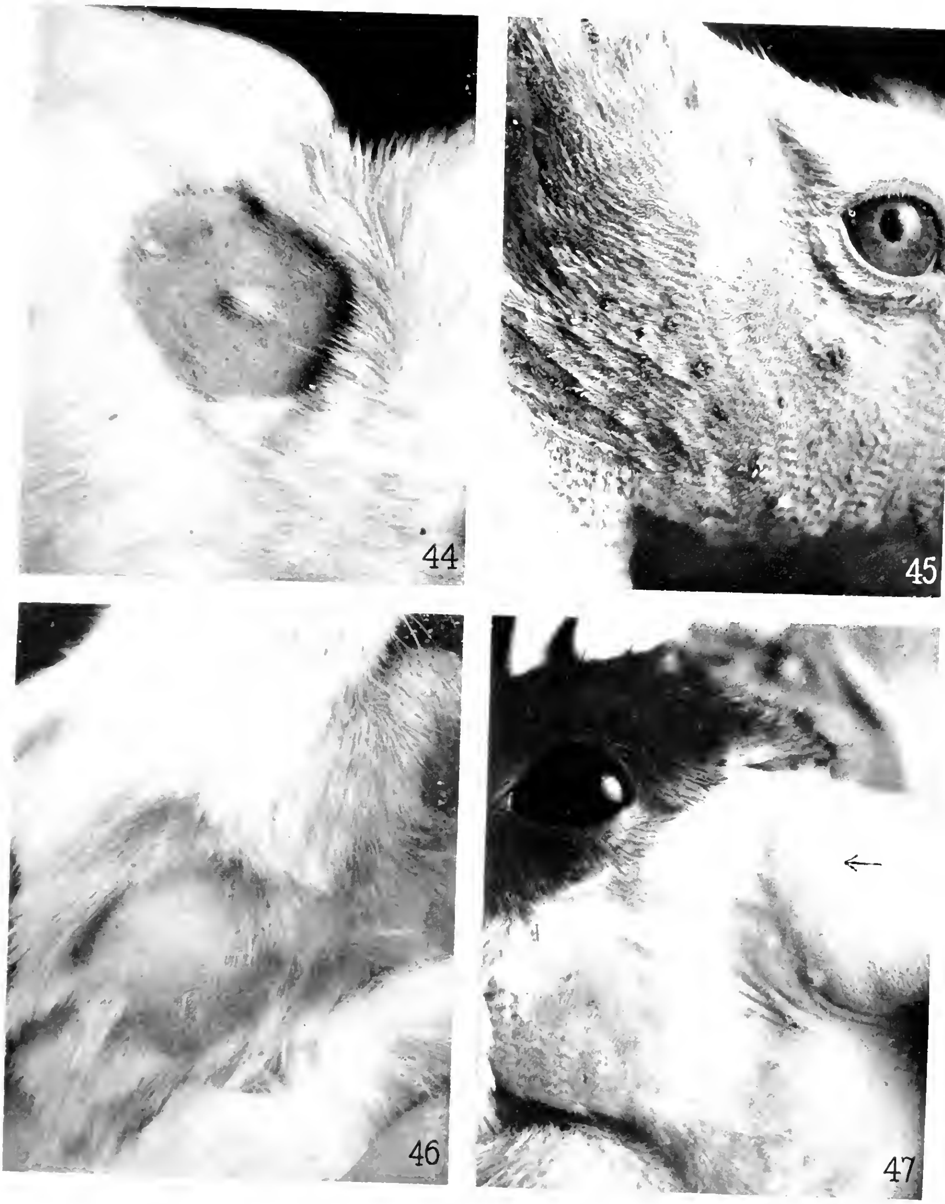
(Brown and Pearce: Experimental syphilis in the rabbit. IV.)



(Brown and Pearce: Experimental syphilis in the rabbit. IV.)



(Brown and Pearce: Experimental syphilis in the rabbit. IV.)



(Brown and Pearce: Experimental syphilis in the rabbit. IV.)

EXPERIMENTAL SYPHILIS IN THE RABBIT.

IV. Cutaneous Syphilis.

Part 2. Clinical Aspects of Cutaneous Syphilis.

By WADE H. BROWN, M.D., and LOUISE PEARCE, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 59 to 77.

(Received for publication, June 14, 1920.)

The character of the cutaneous affections which have been described following local inoculations of *Treponema pallidum* in the rabbit is sufficient to identify these conditions as manifestations of a syphilitic infection and to throw some light upon processes of reaction in the experimental animal. However, the lesions are but the objective signs or the elements of the cutaneous infection, and it will be necessary to give some account of the clinical history of these conditions before a definite place can be assigned to them as integral parts of a generalized infection.

It is the purpose of this paper, therefore, to present such facts concerning the occurrence, distribution, and fate of cutaneous lesions as will enable one to formulate a general conception of cutaneous syphilis in relation to other phases of the experimental infection. In following out this plan of presentation, detailed statements of quantitative relationships will be omitted for the sake of simplicity as well as to avoid any prejudice which might be created from an attempt to assign such values upon the basis of the material which is at present available. Obviously, this aspect of the subject is of importance in itself and will be considered in due time.

Character and Distribution of Cutaneous Lesions in Different Parts of the Body.

From the description of cutaneous lesions which has been given, it is perhaps apparent that these affections tended to preserve a cer-

tain order of distribution and that the character of the lesions present differed somewhat according to their location.

The parts most frequently involved were the hind feet and legs, the head, the front feet and legs, and the tail.[1] This order of distribution was subject, however, to rather wide variations which corresponded in a measure with similar fluctuations in the incidence of different types of cutaneous affections. This is a fact of some importance, since the distribution of the lesions or the character of the lesions present in a given case was undoubtedly influenced to some extent by the particular organism with which the animal was infected. Further than this, the lesions did not occur indiscriminately over those parts of the body which have been mentioned but were confined, for the most part, to a few selected areas.

Lesions of the Head.

Among the animals studied by us, there was a large number which showed involvement of the skin about the head, and the lesions seen in this locality represented practically every form of cutaneous affection which has been described but with a decided predominance of processes of an infiltrative character. The points of especial predilection were certain parts of the face, the brows, the lids, the lips, the base and free portions of the ears, and the cheeks, and the affections peculiar to these areas will be taken up in the order given.

Face.—The lesions which occurred in the skin extending from the nose up over the forehead were chiefly small flattened areas of infiltration or slightly elevated papules, the usual location of which was the sides and bridge of the nose. The appearance presented by animals with affections of this type and the general distribution of the lesions may be illustrated by the photographs reproduced in Figs. 1 to 8. Some of these conditions were so apparent as to be recognized at a glance, but not infrequently there were no visible signs of abnormality and lesions could be detected only by careful palpation of the parts. Thus, the papule on the nose of the animal in Fig. 5 was made conspicuous by its location on a prominent part of the nose and in an area where the hair is short, while a lesion of essentially the same character in Fig. 6 gave no visible sign of its presence.

---

[1] Lesions about the anus and sheath were not included among the cutaneous affections but with those of mucocutaneous borders, while perineal affections were entirely eliminated for reasons which will be stated below.

As a rule, facial lesions were multiple, bilateral, and symmetrical. The more common ones were of the type represented in Figs. 2, 4, 5, and 6. Occasionally, however, affections of a more pronounced character were seen such as those in Figs. 1, 7, and 8. These were usually single and occupied a position at or near the midline in the region of the bridge or sides of the nose.

Brows.—The brows were less often involved than the face but were the seat of a variety of lesions. Those which appeared to be most characteristic of this area were small indurated papules of the type shown in Figs. 9 and 14, together with focal areas of infiltration and necrosis identical with those of the face. Two such lesions are indistinctly seen in Fig. 4. In addition to these, papules of a larger type or even granulomatous lesions were observed in a few animals, a characteristic example of which is given in Fig. 10.

With two exceptions, the lesions which occurred on the brows were bilateral and were usually accompanied by similar conditions on the upper eyelids (Fig. 14).

Eyelids.—Localized infections of the eyelids, while comparatively common occurrences in the rabbit, gave rise to two groups of conditions which could not be clearly separated from one another. In some instances, the lesions arose from the cutaneous surfaces of the lids and appeared to represent affections of essentially the same status as those of the brows; in others, they were clearly marginal in origin and related to the transitional area, or the infection was confined to the conjunctival surface.

However, the general character of the conditions found upon the external surfaces of the lids may be described without attempting to make any sharp distinction as to their origin.

The simplest form of lesion on both the upper and lower lids was a small papular infiltration of the type shown in Fig. 11, and these were especially common in the marginal areas. Fleshy papules measuring several millimeters in diameter were occasionally seen on the upper lids also (Fig. 13), but the usual affection was a small indurated papule with surface ulceration (Fig. 12) or lesions of a papulosquamous type such as those in Fig. 14. These lesions were often bilateral and multiple and were not infrequently associated with similar affections of the brows.

The distinctive feature of the lesions on the lower lids was their size. As a rule, they were larger and of a more fleshy character than those of the upper lids and exhibited a constant tendency to encroach upon the free margin of the lid. Their surface was usually scaly or covered with small crusts (Figs. 15 and 16), and in two instances, large chancre-like lesions were formed which underwent central necrosis and ulceration as shown in Fig. 17.

Lesions of the lower lid were all unilateral and single.

Lips.—There were comparatively few animals which showed localized infections on the lips, with the exception of those in which the lesions were situated along the margins of the nasolabial folds. The only conditions seen were patches of infiltration analogous to those in Fig. 18 or small indurated papules of the

type shown in Figs. 5 and 6. Other conditions affecting the marginal area will be described elsewhere.

The lower lip and chin were very rarely affected. Thus far, we have seen only three animals with lesions in this location, and all of these were comparatively small papules or areas of infiltration one of which is illustrated in Fig. 19.

Base of the Ears.—The skin at the base of the ears and over adjacent parts of the cheeks and neck formed another area for the localization of cutaneous affections (Figs. 20 to 25 and 83 to 88[2]). The lesions seen here were of two very different types and included processes varying from small papular infiltrations analogous to those in Fig. 20 to larger lesions of a circumscribed or diffuse character, (Figs. 21 to 25). In a few instances, large granulomatous masses such as those in Fig. 23 were seen, but the more common affections were small indurated papules or patches of diffuse infiltrations showing various forms of secondary alteration such as were described and illustrated in Part 1 of this paper. Not infrequently these were associated with similar processes on other parts of the ears or tended to spread from the basal area to adjacent parts of the ears as in Fig. 24.

By reference to the photographs illustrating these conditions, it will be seen that the position of greatest predilection for these affections was the lateral surface of the ears immediately below and at the sides of the intertragal incision. The lesions in this area were usually multiple; in some instances they were confined to one side, but in others they exhibited the most perfect bilateral symmetry (Fig. 23).

Ears.—The auricle was also the seat of cutaneous lesions in a number of animals. In the main, these were of the type of macular or small papular eruptions. The macular lesions have been described in detail and no further comment need be made here.

Upon the outer surface of the ears (Figs. 22 and 24 to 27), comparatively few lesions were seen, and, with the exception of that shown in Fig. 26, they were all situated on the lower portion of the ear and occupied one of four positions: the margins of the intertragal incision (Figs. 24 and 27), the medial surface of the ear (Fig. 25), and the area of the anterior or posterior marginal vessels (Fig. 22).

The majority of these lesions were flattened areas of infiltration covered by silvery or grayish yellow scales with occasional points of necrosis or ulceration covered by crusts; in a few instances they were small indurated papules.

Over the internal surfaces, three types of lesions were noted (Figs. 28 to 31). One of these was the macular erythema (Fig. 31) and another the small papular eruption (Fig. 30) which have already been described. These affections occurred chiefly upon the outer part of the ear and more often near the anterior than the posterior margin. They occurred singly, as in Fig. 28, or in groups, and occasionally exhibited a circinate arrangement or appeared to follow the course of the marginal vessels.

---

[2] See Part 1 of this paper also: *J. Exp. Med.*, 1920, xxxii, 445.

A third type of lesion which was seen only once was that shown in Fig. 29. This condition developed as a flattened area of infiltration with slightly elevated margins. The surface was first covered with epithelial scales and later by yellowish gray crusts as shown in the photograph. There was a single lesion of this type on each ear.

Individual lesions on both the internal and external surfaces of the ears varied from a few millimeters to a centimeter or more in diameter and were usually symmetrically placed upon the two ears.

Cheeks and Neck.—With the exception of the area at the base of the ears, there were few lesions seen on the cheeks and neck which could be identified as syphilitic. The small follicular lesions and their various modifications referred to in the preceding paper (Part 1) were the most common conditions observed. In addition, several animals showed a diffuse infiltration of the skin over the cheeks and sides of the neck with a loss of hair and a tendency to desquamation or exfoliation.[3] There was one animal in which the area of involvement extended well down over the shoulder and resulted in the formation of two irregular patches of necrosis.[4] These were the only definite lesions seen in this part of the body. One other animal showed a small flattened lesion of a scaly character in the triangular area at the back of the neck which was probably specific, since at the same time there was a profusion of definite skin lesions elsewhere.

Lesions of the Hind Feet and Legs.

The hind feet and legs of the rabbit hold the distinction of being the most common location of cutaneous lesions (Figs. 32 to 55). This applied especially to granulomatous lesions, and it was here that this type of affection reached its highest state of development. In addition to the granulomata, there were, however, rashes of various types, onychias, and paronychias.

Ordinarily, these affections produced but little change in the general appearance of the parts, and the conditions which one might note were in the main prominent swellings due to the presence of large granulomatous lesions or areas of necrosis and ulceration such as are shown in Figs. 32 to 34. Instances of this kind were comparatively rare, however, and as may be seen by reference to Figs. 35 to 38, which are photographs taken before and after removal of the hair, even marked affections of the skin usually produced only slight changes in appearance.

[3] See the section on alopecia in Part 1 of this paper.

[4] Fig. 8, Part 1.

The lesions on the hind feet and legs were generally located along the lateral margins of the feet more upon the dorsal than the plantar surface, and were distributed from the region of the tendo achillis to the base of the fifth toe. The positions of greatest frequency were the region of the tarsus and external malleolus, the base or tuberosity of the so called fifth metatarsal, the lateral and posterior surfaces of the heel and tendo achillis, and the region of the metatarsophalangeal joint. These peculiarities of distribution and the character of the lesions found in the various locations are brought out by the photographs reproduced in Figs. 36 to 55. It will be noted that the positions enumerated are in a sense pressure points or are skin areas overlying bony or tendinous prominences, and a very large proportion of the cutaneous lesions of the hind feet and legs occurred at these particular points.

Typical examples of the conditions described are illustrated in Figs. 35 to 38 and 39 and 40 which show the hind feet of two animals with marked skin involvement. By reference to the other figures of the series (Figs. 41 to 55), it will be seen that individual variations in the size, number, and location of the lesions were very great. In some instances, frank lesions of the dorsum of the foot or ankle were noted (Figs. 36, 37, and 50), and in their growth those which developed upon the sides of the feet extended in this direction rather than towards the plantar surface. However, plantar lesions or lesions which extended towards the plantar surface were also encountered in some instances (Figs. 51 to 53), and were usually located in the tarsal arch or more exactly in the cushion covering the head of the os calcis just posterior to the base of the fifth metatarsal. Lesions occupying this position are shown in Figs. 49, 51, and 52.

Comparatively few lesions were seen on the legs, and most of these were situated over the tendo achillis, usually not more than a centimeter or so above its attachment to the calcaneus. Occasionally, however, they occupied a much higher position, as in Fig. 46, and in two instances lesions were found on the anterior surface of the shins. One of these is shown in Fig. 36.

The characteristics of distribution described were more closely observed by granulomatous lesions than by those of an infiltrative character. The latter manifested a greater tendency to spread in a linear fashion along the outer side of the foot and might, as in Fig. 54, form an almost continuous line of lesions extending from ankle or heel to the toe, but even here the tendency to localize or to concentrate at certain points was still apparent (Figs. 53 to 55).

As may be gathered from an examination of the accompanying illustrations, lesions of the hind feet and legs were occasionally single

or multiple and unilateral, but in the vast majority of cases they were multiple, bilateral, and symmetrical in their distribution.

After what has been said in regard to similar affections elsewhere, no description of the lesions seems necessary. The illustrations are sufficient in themselves to convey the necessary impressions of the variations in size and character of the several types of lesions to enable one to form a proper conception of the subject.

In addition to the lesions described, mention may be made of the occasional presence of such conditions as onychia and paronychia.

Lesions of the Front Feet and Legs.

The prevailing lesions of the front feet and legs were of essentially the same character as those of the posterior extremities but with a larger proportion of infiltrative processes. The common location of these affections was the extensor and lateral surfaces of the fore arms and the dorsum and sides of the feet as shown in Figs. 56 to 70.

The granulomata were usually situated on the fore arms just above the carpus or on the lateral margins from the level of the carpus to the base of the fifth toe; small multiple lesions were sometimes distributed over the carpus and dorsum of the feet as in Figs. 62 and 65. Occasionally also granulomatous lesions were found at a much higher level on the fore arm (Fig. 64), and in rare instances were located on the flexor instead of the extensor surface (Fig. 59).

The general distribution of the cutaneous infiltrations was much the same as that of the granulomata, but they differed in certain respects. In the case of the infiltrations, the lesions usually appeared along the ulnar margins of the fore arm and carpus instead of the extensor surfaces; they almost invariably extended over the dorsum and sides of the feet (Figs. 66 to 73) and frequently involved the median as well as the lateral surfaces (Figs. 69 and 71). In one instance, large crustaceous lesions were formed about the elbow (Figs. 74 and 75).

The cutaneous granulomata not infrequently appeared as single lesions of a large size or as multiple affections of one fore arm. In contrast to this, the infiltrations were in all cases multiple affections and bilaterally symmetrical in their distribution.

Other conditions affecting the fore paws were onychia and paronychia which were most common on the outer or fifth toe (Figs. 74 and 75), but in several animals other toes as well were involved (Fig. 71).

In connection with lesions of the fore arms, attention may be called to the frequent occurrence of edema during periods of marked activity on the part of

various types of cutaneous lesions, since examples of this condition may be seen by reference to Figs. 59 and 61. In the first of these, a very pronounced swelling about the carpus and foot can be made out, and in Fig. 61, there is a marked edema of the skin and subcutaneous tissues which extends beyond the middle of the fore arm.

In other instances where no edema was present, it was noted that shaving an area where there was syphilitic involvement or the infliction of slight trauma by other means which had no effect upon the skin of a normal animal was frequently sufficient to cause a rapid development of edema which lasted sometimes for days. This urticarial reaction of the skin, if it may be termed such, was not peculiar to cases of cutaneous infection but occurred also in animals in which the lesions were situated in the deeper structures, as in the bones. It suggests, therefore, the action of some toxic influence upon the vascular mechanism of parts adjacent to foci of active syphilitic infection, and this feature of the infection is worthy of consideration in connection with the various cutaneous and other reactions which occur subsequent to the administration of therapeutic agents.

Lesions of the Tail.

Localized infections of the tail form a group of conditions which is somewhat obscure. Uhlenhuth and Mulzer (1) reported "tail tumors" as a frequent occurrence following generalized inoculation of rabbits, and the condition thus described was in most instances a bulbous swelling of the distal end of the tail. Except in this connection, we have seen no mention of tail lesions in the literature. Such conditions do occur, however, and are probably of rather frequent occurrence, but the difficulties surrounding an examination of this part of the body are so great that we were unable to give it the attention which it probably deserved and hesitate, therefore, to commit ourselves as to the frequency and importance of this class of affections.

Again, we have been able to recognize two conditions affecting the skin of the tail (Figs. 76 to 82). The one most commonly observed was a granulomatous lesion generally involving the ventral surface or sides of the tail, rarely the dorsal (Figs. 76 to 81). As may be seen in the accompanying illustrations, these lesions were either single or multiple, and while they occurred at various levels, they were

more often found on the proximal and middle thirds than towards the outer end of the tail.

The second type of affection was that shown in Fig. 82. In these cases, the skin involvement was of an equally marked character, especially towards the end of the tail, but partook more of the nature of a diffuse infiltration. The photograph reproduced in Fig. 82 shows a thinning of the hair and the presence of bald patches over the ventral surface of the tail which characterized this group of affections. Towards the outer end, there is also a small area of necrosis. In one case of this kind, the skin covering the entire tail underwent necrosis and sloughed away.

The six cases of cutaneous syphilis of the tail which have been used for illustration are such as might be recognized without any considerable difficulty, and it will be noted that none of them conforms to the "tail tumor" type of lesion. Bulbous expansions of the end of the tail were observed, however, in a few animals but were always of a relatively slight degree and were difficult to detect because of the fact that the physical alterations present were within the range of normal variations in tail structure. These variations are so great that unless one has an accurate record of the tail of each animal before inoculation or the pathological alterations which take place are quite marked, little can be determined by palpation and the fur must be removed before inspection will be of any material assistance. These procedures we were unable to carry out as a routine part of our work and we are not in a position, therefore, to speak with any degree of assurance as to conditions other than those observed.

Lesions of the Trunk.

With the exception of the perineum, few instances of cutaneous lesions involving the trunk have come under our observation. As previously stated, alopecia was noted in a number of animals, and after removal of the hair from localized areas some peculiar conditions were observed which may or may not have been syphilitic.[2] This is not to be interpreted as evidence that this portion of the body remains uninvolved. On the contrary, it is known that lesions may occur upon the trunk, since a pronounced eruption on the back was one of the conditions described by Grouven (2) in the first case of generalized syphilis reported. It does mean, however, that under

ordinary circumstances, marked involvement such as one frequently observes in other parts of the body is far less common on the trunk. It should be pointed out also that the regions in which minor lesions were most frequently observed were those which were most accessible to examination, and when it is recalled that some of these lesions are extremely inconspicuous, it seems not unlikely that many such affections might escape detection altogether if situated upon the more thickly covered portion of the body. The occurrence of cutaneous lesions of the trunk must be regarded, therefore, as problematical and a field for future investigation.

As regards the perineum, it may be said that lesions in this locality, including those of the genitalia as well as the surrounding skin surfaces, were quite numerous and included two classes of affections, one representing conditions of cutaneous origin and the other affections of the mucocutaneous borders.

The first of these groups comprised lesions which for the most part were closely connected with the seat of inoculation, and we have avoided placing too much stress upon them lest some confusion might arise between extensions from primary lesions and generalization of the infection as it is commonly understood. However, the descriptions of scrotal lesions given in the preceding papers (3–6) will apply equally well to those which arise in this area as a result of generalization of the virus. Other lesions of the perineum will be described in connection with affections of mucocutaneous borders.

Clinical History of Cutaneous Lesions.

An exact statement of the time of occurrence, the duration, and the relative frequency of different types of cutaneous lesions in the rabbit can hardly be given without entering into a detailed analysis of the experimental conditions under which the lesions developed, and must be deferred, therefore, until the subject can be approached from this standpoint. There are, however, certain facts concerning the clinical history of these affections which may be recorded, some of them in greater detail than others.

Cutaneous Eruptions.—The cutaneous eruption in the rabbits usually consisted of only a few lesions occurring singly or in small

groups upon some one part of the body such as the head or the feet and legs. Occasionally, the lesions were more numerous and more widely distributed, several parts of the body being involved at about the same time or in rapid succession to one another.

The order in which the lesions appeared was somewhat irregular. It was not uncommon for several or all of them to make their appearance at about the same time. In other instances, one or two lesions developed, and no others were detected for a week or more when new lesions appeared and were followed by others at rather long and irregular intervals which at times extended over several months, or, after the appearance of the first lesions, others developed at short intervals until the eruption was complete.

How many distinct periods of eruption might occur is impossible to say, since the majority of the animals were not held for any great length of time. Among those which were kept under observation for a year or more, some showed only a single period of cutaneous eruption, while in others, there were several such periods separated by long or short intervals during which no lesions were present or no new lesions appeared.

In some instances, the consecutive eruptions consisted of lesions situated in places not previously involved, while in others, they might be regarded more as relapses than as new eruptions, since the new lesions developed at the site of older ones.

It was usually but not invariably the case that the lesions of the first eruption were of a larger type and more numerous than those of succeeding crops. This applied especially to the granulomata, an instance of which may be seen by comparing Figs. 39 and 40 with Figs. 41 and 42. Figs. 39 and 40 represent the first cutaneous eruption on the hind feet. These lesions healed completely and remained healed for between 4 and 5 months at which time two of them recurred (Figs. 41 and 42).

Another very important feature of the cutaneous eruption was the apparent influence exerted by one lesion or group of lesions upon another. This was best shown in instances where multiple lesions occurred in the same locality, as upon the fore arms and wrists of the animal in Figs. 62 and 63. When, as in this case, a number of lesions made their appearance at about the same time, the growth of most of

them was abortive, and only one or two developed to any considerable extent. It may be recalled that this same condition was pointed out in connection with the development of multiple lesions of the scrotum.

The inhibition thus exerted by one focus of reaction upon another is a phenomenon of the most fundamental importance and furnishes the key to an explanation of many conditions which characterize the experimental infection. In the present instance, it probably accounts for the existence of so few lesions and the unusual size which individual lesions frequently attain.

From this description of cutaneous eruptions, it is apparent that they were exceedingly variable as to the number and character of the lesions present at a given time, the manner of their appearance, and the number of eruptions which might occur in a given case.

Time of Occurrence of Cutaneous Lesions.—Cutaneous lesions were among the earliest manifestations of a generalized infection, but the time of their appearance was subject to very wide variations. This appeared, however, to be partly due to differences in experimental conditions. The first cutaneous eruption usually appeared between the 2nd and 4th months after inoculation and on the whole was earlier in the case of the granulomata than with lesions of an infiltrative character. The earliest recorded time for the appearance of cutaneous lesions was 3 weeks after inoculation, and the longest interval between inoculation and the first cutaneous eruption was 2 years and 8 months; the next longest was between 6 and 7 months. It may be said, therefore, that the first cutaneous eruption appeared at from 6 weeks to 6 months after inoculation with occasional cases occurring earlier or later, depending upon the conditions of the infection.

As regards the occurrence of subsequent eruptions, or the interval between eruptions, very little can be said. In the few animals which were held for any considerable length of time, the period of active eruption rarely extended beyond 4 to 6 months after inoculation. One case has been mentioned, however, in which the first lesions were noted 2 years and 8 months after inoculation. A second eruption occurred in this animal 3 years and 5 months after inoculation, and a third eruption 2 months later. Lesions of this second group are shown in Fig. 52.

Two other animals of our series showed repeated periods of active cutaneous eruption extending over more than 2 years from the time of inoculation, but during this time they were never entirely free from lesions. A third animal now under observation has followed much the same course for a period of 8 months. In a fourth animal, as in the case of the one cited above, there were three sharply separated periods of eruption spaced at intervals of approximately 7 months. These few cases will serve to indicate the difficulty in attempting to set any time limits upon the occurrence of cutaneous lesions when so few animals were kept under observation for any considerable period of time, but they also indicate that cutaneous affections may occur during the late as well as the early stages of the infection.

Clinical Course of Cutaneous Lesions.—The clinical course of the cutaneous infection was essentially the same as that of the testicular or scrotal lesions. The tendency to pursue a periodic or relapsing course was apparent in the development and resolution of individual lesions as well as in the recurrence of healed lesions and the development of successive crops of eruptions.

These features of the skin reaction were again most evident in the granulomata, the growth of which usually proceeded by irregular stages interrupted by periods of inaction or even regression. Relapse of partially or completely healed lesions, which is but an exaggerated form of this reaction, was a comparatively common occurrence and may be illustrated by the series of photographs reproduced in Figs. 83 to 88 representing the state of a lesion at intervals of 90, 97, 105, 133, 141, and 160 days respectively after inoculation. This type of phenomenon was practically constant, but we have little evidence upon which to base an estimate of the frequency of relapse of completely healed lesions. It may be mentioned, however, that of the few animals kept under observation for a year or more, a number showed recurrence, and with three of them there were several periods of complete healing of individual lesions followed by relapse.

Duration of Cutaneous Lesions.—Spontaneous regression and healing were the common fate of all cutaneous lesions, but great differences were found in the duration of different types of lesions as well as of individual lesions. As was mentioned elsewhere, the macular erythemata were characteristically of short duration, sometimes dis-

appearing within 24 to 48 hours and rarely persisting for more than a few days. In like manner, small infiltrations not infrequently disappeared spontaneously within a few weeks after they were first noted.

On the other hand, granulomatous lesions and many of the infiltrations, especially the papular lesions of the brows and lids, were more enduring as a rule. For example, the papular lesions of the brows and lids shown in Fig. 14 lasted more than a year before they completely disappeared, and the lesion at the base of the toe in Fig. 48 is one of a small group which showed an even greater persistence, having lasted for more than 2 years. These were exceptional cases, however, and from the data available, it would appear that the average duration of cutaneous lesions was hardly more than 2 to 4 months, although in a fair percentage of animals, this period might be prolonged by several months.

The duration of active skin infection was somewhat longer than that of the individual lesion, since all the lesions present did not pursue a parallel course of changes. The available data bearing upon this feature of the infection, however, are insufficient to permit of an estimation of the time which such infections might endure.

Detection and Diagnosis of Cutaneous Lesions.

In view of the great frequency with which cutaneous lesions appear to follow local inoculations of *Treponema pallidum* in the rabbit and the fact that so few of these conditions have been described, it seems well to refer briefly to the detection and diagnosis of this class of affections.

The first essential to the detection and diagnosis of cutaneous lesions is obviously a knowledge of the character and distribution of these affections, and this we have attempted to supply. Large granulomatous lesions such as those in Figs. 32 to 34 which produce striking irregularities in the contour of the affected parts, or lesions which have resulted in a considerable destruction of the skin surface, as in Figs. 74 and 75, may be seen almost at a glance if one knows where to look for them. Prominent swellings and surface erosions are not always present, however, and at all events are late phenomena. The great majority of the lesions which affect the feet, legs, and tail are

concealed from view and can be detected only by palpation, rarely by inspection. Lesions about the head are more exposed as a rule, and careful inspection of the regions in which they are known to occur is highly essential, but with the exception of a few areas such as the ears and the eyelids, even here the chief reliance for the early detection of cutaneous lesions must be placed upon palpation.

The technique of this operation consists in picking up the skin and gently rolling it between the thumb and finger. In this way, one is soon capable of detecting the slightest thickening or irregularity in the skin, and if the hair is removed and these initial changes are carefully followed from day to day, little difficulty will be experienced in arriving at a diagnosis.

If the animals are separately caged and well cared for, few conditions will arise which are apt to be confused with typical syphilitic lesions, even the smaller infiltrations and erosions. Apart from diseases of the skin, such as mange, and traumatism inflicted by the animal itself, which are very readily recognized, the only three conditions which need be considered are small focal abscesses, old scars, and focal thickenings due to a new growth of hair. The first two conditions are, in the main, trunk affections and should not be confused with syphilitic lesions. The third condition may occur anywhere and is especially troublesome about the face, but clipping of the hair and careful observation will usually enable one to arrive at a correct diagnosis. Examination for spirochetes may or may not prove helpful in these cases and may even defeat the object of the examination by causing regression of a syphilitic lesion before a characteristic condition has been established.

CONCLUSIONS.

The description of cutaneous syphilis in the rabbit following local inoculation is incomplete in many respects but is sufficient to indicate that a generalized infection of the skin does occur in a large number of animals—just how often and under what circumstances we shall not attempt to say.

It is apparent, however, that the cutaneous affection is a very characteristic one. The lesions themselves bear the marks of a varied

but definite pathological process identical in all respects with the reaction set up in the scrotum by local inoculation of *Treponema pallidum.* The character of the lesions and, withal, their great multiformity, the time relations existing between inoculation and the appearance of the cutaneous lesions, the occurrence of successive crops of eruptions, the relapsing course of the disease, and the preservation of a fixed order of distribution of the lesions, are highly suggestive of cutaneous syphilis in man, and it is believed that as more is known of the experimental infection and with better adaptation of organisms, the analogy will become even closer.

SUMMARY.

From the study of a large number of rabbits with generalized cutaneous syphilis following local inoculation with *Treponema pallidum,* lesions were found most often about the hind feet and legs, the head, the front feet and legs, and the tail. There was further evidence of a selective distribution of cutaneous lesions in the fact that, on a given part of the body, the lesions were usually confined to a few restricted areas. About the head, they occurred almost exclusively on the sides and bridge of the nose, the lids, the brows, the lips, and the base and free portions of the ears. On the front feet and legs, the seat of predilection was the extensor and lateral surfaces of the fore arm, the carpus, and the feet, while on the posterior extremities they were situated upon the dorsum and lateral surfaces of the feet and ankles from the level of the tendo achillis to the base of the fifth toe. The positions of greatest frequency were the region of the tarsus and external malleolus, the base of the fifth metatarsal, the lateral and posterior surfaces of the heel and tendo achillis, and the base of the fifth toe. In many instances, the positions of predilection were exposed positions or areas of skin covering bony or tendinous prominences.

It was also found that the character of the lesions differed somewhat in the various locations. The lesions of the head were mostly small circumscribed papules or processes of diffuse infiltration; on the fore arms and feet, affections of this type were about equally divided with larger granulomatous masses of a chancre-like character, while on

the hind feet and legs, granulomatous lesions were far more numerous than those of any other type and frequently reached a very large size.

The cutaneous eruption usually consisted of only a few lesions confined to some one part of the body, but occasionally they were more numerous and more widely distributed. In this connection, it was noted that when multiple lesions appeared in a given area at about the same time, the growth of most of them was abortive, and, as a rule, only one or two developed to any considerable size. Especial emphasis was placed upon this phenomenon of inhibition as a factor of fundamental importance in the experimental infection.

From clinical observation, it was found that, as a rule, the first cutaneous eruption occurred at from 2 to 4 months after inoculation but might occur either earlier or later, depending upon the circumstances in the individual case. The earliest eruptions appeared 3 weeks after inoculation and the latest 2 years and 8 months, but, as a rule, the time between inoculation and the appearance of the first eruption did not exceed 4 to 6 months.

Successive crops of cutaneous lesions appeared in a number of animals usually within the first 6 months after inoculation. In a few instances, however, there were repeated eruptions extending over a period of 2 years or more, the longest recorded period being 3 years and 7 months.

The duration of individual lesions was found to be extremely variable, ranging from a few days in the case of a macular erythema to more than 2 years in the case of a few granulomatous lesions. The average duration of the lesions appeared to vary somewhat with the nature of the lesion but on the whole was not more than 2 to 4 months. No limits could be fixed, however, for the duration of an active skin infection as a whole.

Again, it was found that the cutaneous infection tended to pursue a periodic or relapsing course. This was seen in the mode of growth and resolution of individual lesions, the occurrence of successive periods of eruption, and the recurrence of completely healed lesions, all of which was interpreted as evidence of the essential relapsing nature of syphilitic infections.

BIBLIOGRAPHY.

1. Uhlenhuth and Mulzer, P., *Arb. k. Gsndhtsamte*, 1910, xxxiv, 222.
2. Grouven, C., *Dermat. Z.*, 1908, xv, 209.
3. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1920, xxxi, 475.
4. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1920, xxxi, 709.
5. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1920, xxxi, 729.
6. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1920, xxxi, 749.

EXPLANATION OF PLATES.

With the exception of Fig. 31 the illustrations are from unretouched photographs which represent the objects at their natural size. The time given is estimated from the date of inoculation unless otherwise stated.

Plate 59.

Figs. 1 to 4. Clinical appearance of animals with syphilitic lesions about the face.

Fig. 1. 58 days. An extensive infiltration of the frontal region showing loss of hair and exfoliation over the affected area.

Fig. 2. 138 days. Multiple foci of infiltration and necrosis distributed over the sides and bridge of the nose. There was a similar condition on the left side of the face.

Fig. 3. 249 days. A diffuse area of infiltration with some loss of hair and desquamation of epithelium involving a portion of the lip and the skin over the side of the nose.

Fig. 4. 188 days. Multiple focal lesions over the lower portion of the nose, the right upper lip, and the brows. Lesions were also present about the nares.

Plate 60.

Figs. 5 to 10. Syphilitic lesions of the face and brows.

Fig. 5. 69 days. Early papular lesion on the side of the nose.

Fig. 6. 98 days. Small nodular lesion, side of the nose, which was not visible until the hair was removed.

Fig. 7. 128 days. Circumscribed nodular lesion with central necrosis, situated on the lower portion of the bridge of the nose.

Fig. 8. 169 days. Extension and transformation of the lesion in Fig. 7.

Fig. 9. 138 days. Small papular lesions with apical necrosis on the brow.

Fig. 10. 112 days. Large nodular syphilide with central necrosis and ulceration on right brow.

Plate 61.

Figs. 11 to 19. Syphilitic lesions of the lids, brows, and lips.

Fig. 11. 98 days. Early papular lesions on upper and lower lids. There are two small lesions on the margin of the upper lid and a single larger lesion on the lower lid.

Fig. 12. 108 days. Small indurated papule with surface ulceration on the margin of the upper lid.

Fig. 13. 134 days. Large papular syphilide, upper lid, with slight necrosis at the center and considerable desquamation of surface epithelium.

Fig. 14. 13 months. Multiple lesions of the upper lid and brow. These lesions were of the nature of small papular infiltrations covered by heavy epithelial scales.

Fig. 15. 80 days. Large nodular lesion, lower lid. This lesion was of an intense copper color with small patches of scales over its surface.

Fig. 16. 113 days. Nodular lesion of the lower lid similar to that in Fig. 15, showing foci of necrosis covered by crusts.

Fig. 17. 84 days. Large granulomatous lesion of the lower lid. The center is necrotic and covered by a thick crust.

Fig. 18. 65 days. Circumscribed area of infiltration, right upper lip. The surface of the lesion is necrotic and covered by a crust.

Fig. 19. 8 months. Multinodular area of infiltration on the chin. This lesion was of a deep copper color and showed several areas of exfoliation.

Plate 62.

Figs. 20 to 23. Syphilitic lesions of the ears, basal region.

Fig. 20. 53 days. Two small areas of infiltration in the skin marked by arrows. These lesions developed no further than the condition here shown.

Fig. 21. 113 days. Multiple nodules in the skin at the base of the ear.

Fig. 22. 101 days. Small circumscribed area of infiltration with central necrosis just below the intertragal incision. A second lesion is seen posterior to this and a third on the anterior surface of the ear. All these lesions showed a central area of ulceration and a marked tendency to accumulation of epithelial scales.

Fig. 23. 128 days. Large granulomatous lesions at the base of the ears showing bilateral symmetry of this class of affection. A few small areas of infiltration are also seen above the nodular lesions on the left ear.

Plate 63.

Figs. 24 to 29. Syphilitic lesions on the free portions of the ears.

Fig. 24. 8 months. The same animal as in Fig. 22. Spreading patches of infiltration on the lateral surface of the ear.

FIG. 25. 8 months. The same animal. Syphilitic lesions on the median surface of the ear. One of these was a very small area of infiltration (marked by arrow). The other shows an area of necrosis surrounded by a zone of infiltration.

FIG. 26. 104 days. Indurated nodular lesion on the anterior and outer surface of the ear.

FIG. 27. 13 months. Diffuse area of infiltration with exfoliation and necrosis spreading along the anterior margin of the ear.

FIG. 28. 60 days. Circumscribed nodular lesion on the inner surface of the ear, at an early stage of its development.

FIG. 29. 140 days. Circumscribed area of infiltration with raised edges and necrotic center situated on the anterior margin of the inner surface of the ear.

PLATE 64.

FIG. 30. 97 days. Multiple papular lesions on the inner surface of the ear showing circinate arrangement and bilateral symmetry.

FIG. 31. 132 days. Erythematous patches in the ears showing bilateral symmetry.

PLATE 65.

FIGS. 32 to 34. Clinical appearance presented by animals with large granulomatous lesions of the hind feet.

FIG. 32. 122 days. There are four large granulomatous nodules along the outer side of the right foot; on the left, there is a single large lesion in the region of the metatarsal and a second on the outer side of the heel.

FIG. 33. 122 days. Lateral view of the right foot of the same animal.

FIG. 34. 177 days. The same foot at a later date showing the appearance presented after extensive necrosis and ulceration had taken place.

PLATE 66.

FIGS. 35 to 38. Nodular or granulomatous lesions of the hind feet and legs.

FIG. 35. 69 days. Anteroposterior view before removal of the hair.

FIG. 36. 81 days. Appearance presented at a slightly later date with hair removed. The nodular swellings over the outer portions of the fifth metatarsals are due to periosteal lesions.

FIGS. 37 and 38. Lateral views of the same feet. Note especially the character and distribution of the lesions.

PLATE 67.

FIGS. 39 to 42. Nodular or granulomatous syphilides of the hind feet and legs.

FIGS. 39 and 40. 115 days. Right and left hind feet of the same animal showing the character and location of lesions and bilateral symmetry.

Figs. 41 and 42. 10 months. Hind feet of the same animal showing recurrent lesions at the base of the fifth metatarsals. Note the relative size of the lesions as compared with the original lesions in Figs. 39 and 40.

Plate 68.

Figs. 43 to 46. Granulomatous lesions of the hind feet and legs.

Figs. 43 and 44. 154 and 148 days respectively. Right and left hind feet of the same animal showing single chancre-like lesions, one over the external malleolus and the other on the lateral and posterior surface of the tendo achillis just above the heel.

Fig. 45. 76 days. Granulomatous lesions over the tendo achillis, side of the heel, and base of the fifth metatarsal.

Fig. 46. 125 days. Large granulomatous lesion situated high up on the lateral and posterior surface of the tendo achillis.

Plate 69.

Figs. 47 to 50. Atypical distribution of granulomatous lesions on the hind feet.

Figs. 47 and 48. 2 years and 2 months. The same animal. Single granulomatous lesions involving the heel of the left foot and the base of the fifth toe on the right. These lesions are the same as those shown in Figs. 32 to 34.

Figs. 49 and 50. Left and right hind feet of the same animal.

Fig. 49. 91 days. There are four lesions on this foot, one over the external malleolus, a second on the plantar arch, the third on the dorsal surface of the fifth metatarsal, and the fourth at the base of the outer toe.

Fig. 50. 83 days. There are also four clearly defined lesions on this foot, all of which occupy positions on the dorsum of the foot, which is quite unusual.

Plate 70.

Figs. 51 to 55. Plantar lesions and lesions of an infiltrative character on the hind feet and legs.

Fig. 51. 75 days. Large granulomatous lesion on the outer and plantar surfaces of the tarsus and a smaller lesion over the tuberosity of the fifth metatarsal. There is a third lesion on the plantar surface at the base of the fifth toe.

Fig. 52. Lesions identical with those in Fig. 51. The point of chief interest is the time of their occurrence which was 3 years and 5 months after inoculation.

Fig. 53. 68 days. Discrete infiltrations over the lateral surface of the foot with a tendency towards a plantar position.

Figs. 54 and 55. 15 months. Hind feet of the same animal. Multiple infiltrations distributed over the lateral margin of the foot. In Fig. 54, the lesions show necrosis, some exfoliation, and a tendency to fuse with one another.

Plate 71.

Figs. 56 and 57. Nodular or granulomatous lesions of the front feet and legs.

Fig. 56. 81 days. Multiple nodular lesions over the extensor and lateral surfaces of the fore arms and feet. Note character and location of the lesions.

Fig. 57. 69 days. The same feet at a slightly earlier period before removal of the hair.

Plate 72.

Figs. 58 to 61. Lateral views of the same animal as that in Figs. 56 and 57. Note the presence of a lesion on the flexor surface of the fore arm and the edematous swelling shown about the carpus and foot in Fig. 59 and extending to the middle of the fore arm in Fig. 61.

Plate 73.

Figs. 62 to 64. Nodular and granulomatous lesions of the fore arms and feet.

Fig. 62. 113 days. Early multinodular affection with lesions on the extensor surface of the fore arms, carpus, and dorsum of the feet.

Fig. 63. 128 days. Later stage in the development of the same lesions. Note the disappearance of most of the lesions shown in Fig. 62 and marked development of single lesions on each fore arm.

Fig. 64. 84 days. Large chancre-like lesion on the extensor surface of the fore arm situated at an unusually high level.

Fig. 65. 60 days. Early nodular syphilides, dorsum and lateral surface of the foot. The prominent swelling over the carpus was due to a periosteal lesion.

Plate 74.

Figs. 66 to 70. Infiltrative lesions on the fore arms and feet.

Figs. 66 and 67. 202 days. Right and left front feet and legs of the same animal. The lesions here shown were in the beginning parchment-like areas of infiltration (Fig. 66) which underwent necrosis and ulceration (Fig. 67).

Fig. 68. 138 days. Appearance presented by an early lesion similar to those in Figs. 66 and 67. The presence of a lesion is suggested by partial loss of hair and discoloration of the skin shown on the lateral surface of the carpus.

Fig. 69. 157 days. Diffuse infiltration on fore arm, carpus, and dorsum of the feet. Hair clipped.

Fig. 70. 184 days. Lateral view of the lesion in Fig. 69, taken during a period of marked activity.

Plate 75.

Figs. 71 to 75. Cutaneous infiltrations, front feet and legs.

Fig. 71. 15 months. Frontal view of the feet to show lesions present over the median surface of the toes.

Figs. 72 and 73. The same as Fig. 71, showing lesions on the lateral surface of the fore arm and at the base of the toes.

Figs. 74 and 75. 8 months. There is an area of widespread infiltration over the elbows with surface necrosis, exfoliation, and the formation of weeping patches; there is also a well marked paronychia of the fifth toe on both sides.

Plate 76.

Figs. 76 to 82. Syphilitic lesions of the tail.

Fig. 76. 77 days. Early multiple granulomatous lesions on the ventral and lateral surface of the tail.

Fig. 77. 76 days. A large granulomatous lesion near the base of the tail with diffuse infiltration of the skin over the outer portion indicated by the glistening character of the surface.

Fig. 78. 65 days. Multiple lesions of a nodular character on the distal portion of the tail. There are beginning necrosis and ulceration of the largest of the lesions.

Fig. 79. 76 days. The same animal, showing extension of the tail involvement together with necrosis and ulceration of the affected area.

Fig. 80. 113 days. Circumscribed nodular lesions, middle and outer third of the tail. The scar of a former lesion is seen in the area marked with an arrow.

Fig. 81. 68 days. A group of three nodular lesions situated on the ventral and lateral surfaces near the base of the tail.

Fig. 82. 109 days. A diffuse infiltrative process involving the skin of the tail over its entire extent. Note the alopecia over the ventral surface of the tail and a small area of necrosis and ulceration near its distal extremity.

Plate 77.

Figs. 83 to 88. Periods in the history of a cutaneous lesion.

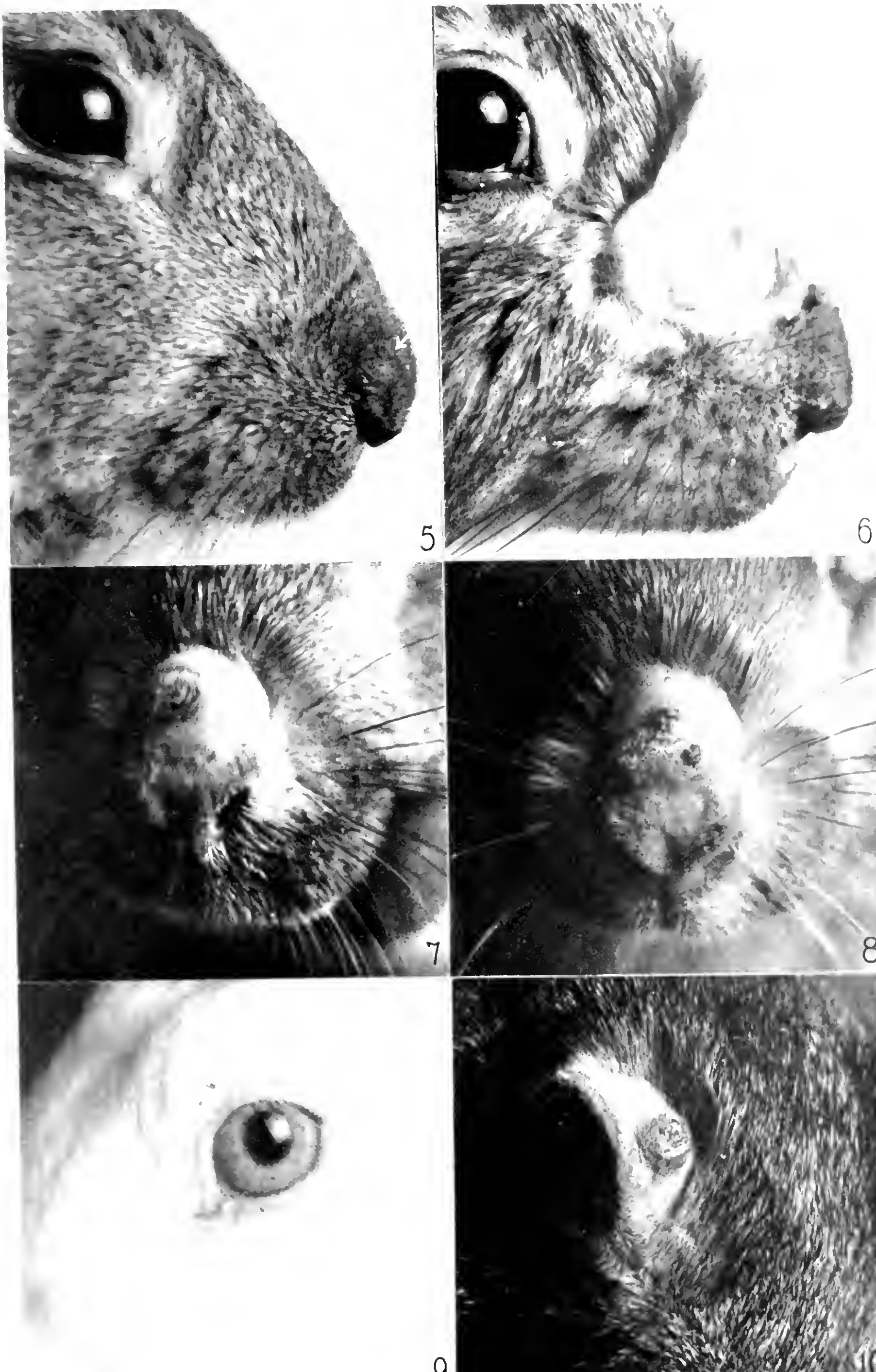
Figs. 83 to 85. Stages of active development of the lesion. 90, 97, and 105 days.

Fig. 86. 133 days. Marked regression of the lesion.

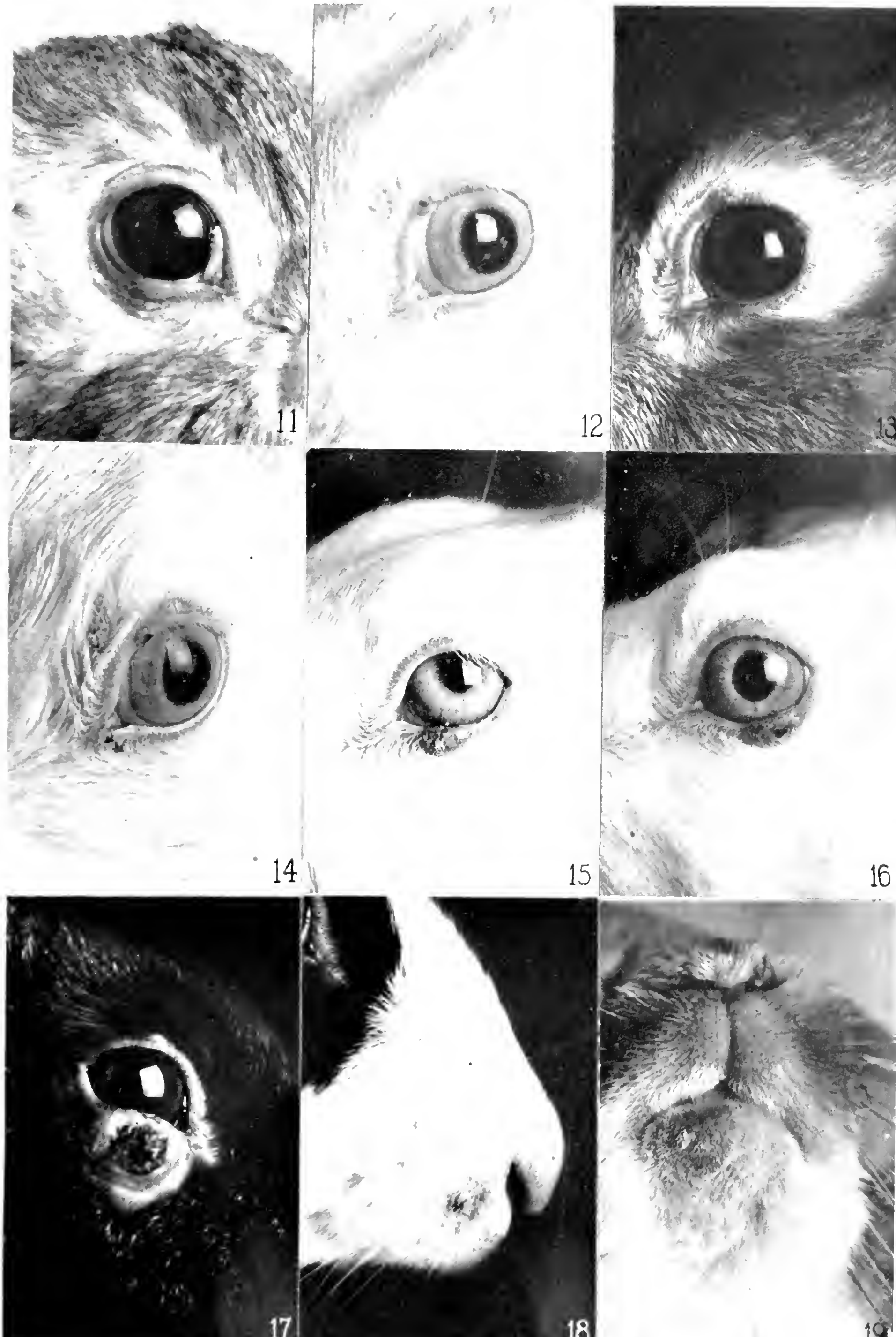
Fig. 87. 141 days. Definite renewal of activity.

Fig. 88. 160 days. A further stage in the growth of the lesion.

(Brown and Pearce: Experimental syphilis in the rabbit. IV.)

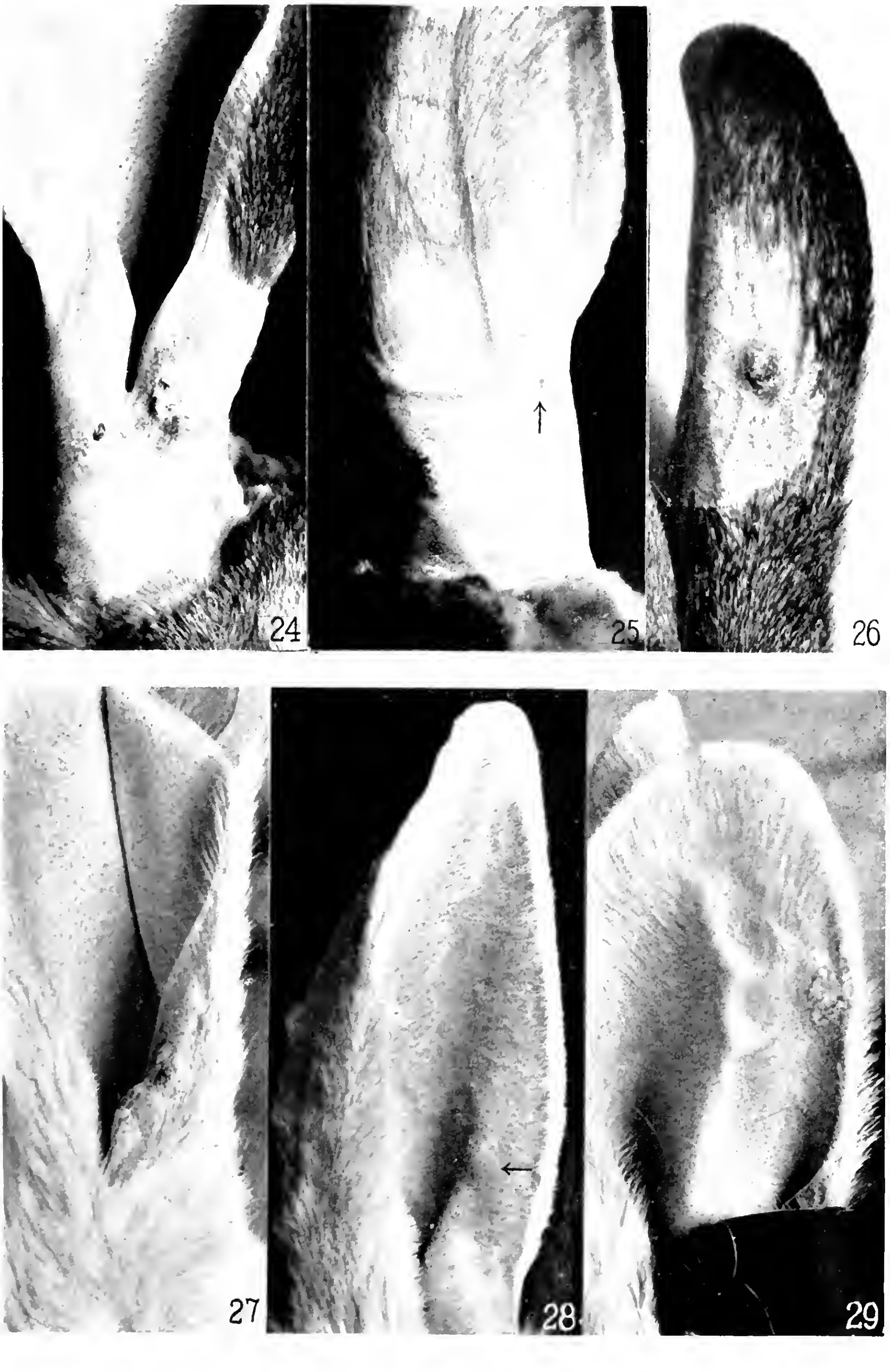


(Brown and Pearce: Experimental syphilis in the rabbit. IV.)

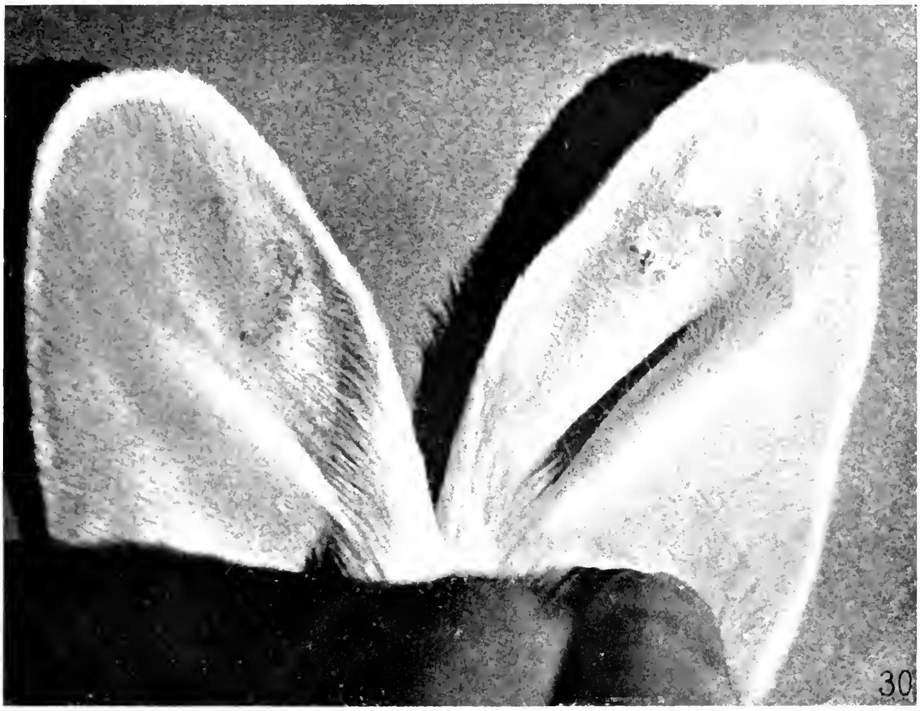


(Brown and Pearce: Experimental syphilis in the rabbit. IV.)

(Brown and Pearce: Experimental syphilis in the rabbit. IV.)



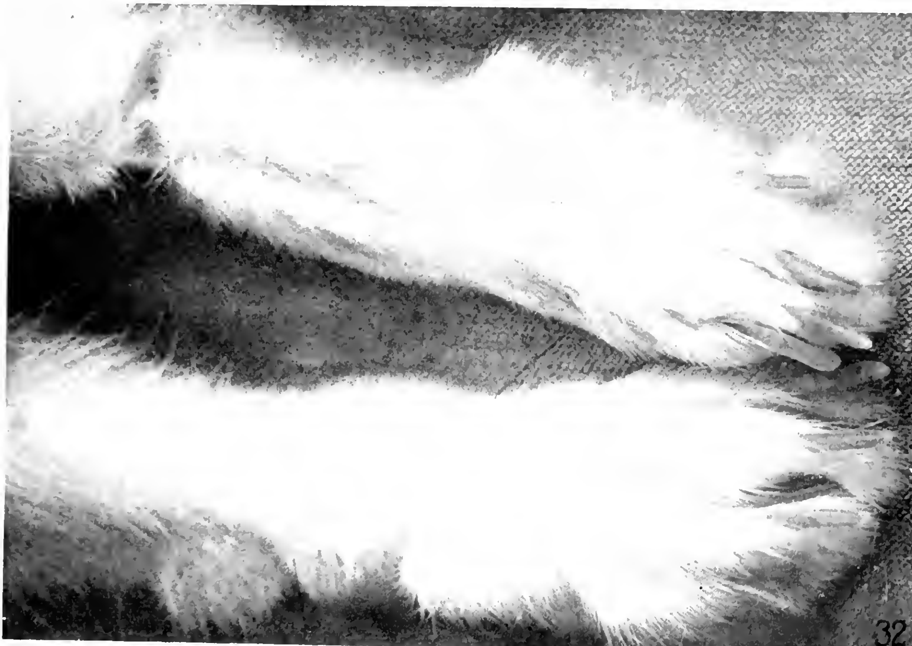
(Brown and Pearce: Experimental syphilis in the rabbit. IV.)



30

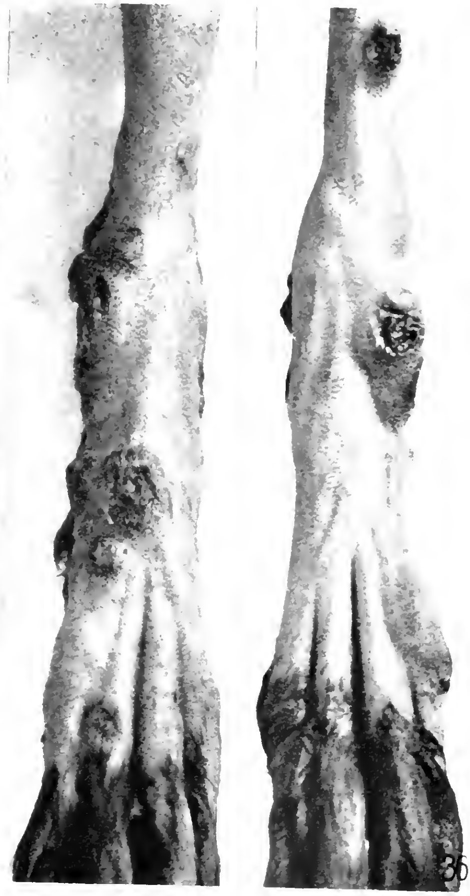
31

(Brown and Pearce: Experimental syphilis in the rabbit. IV.)



(Brown and Pearce: Experimental syphilis in the rabbit. IV.)

35

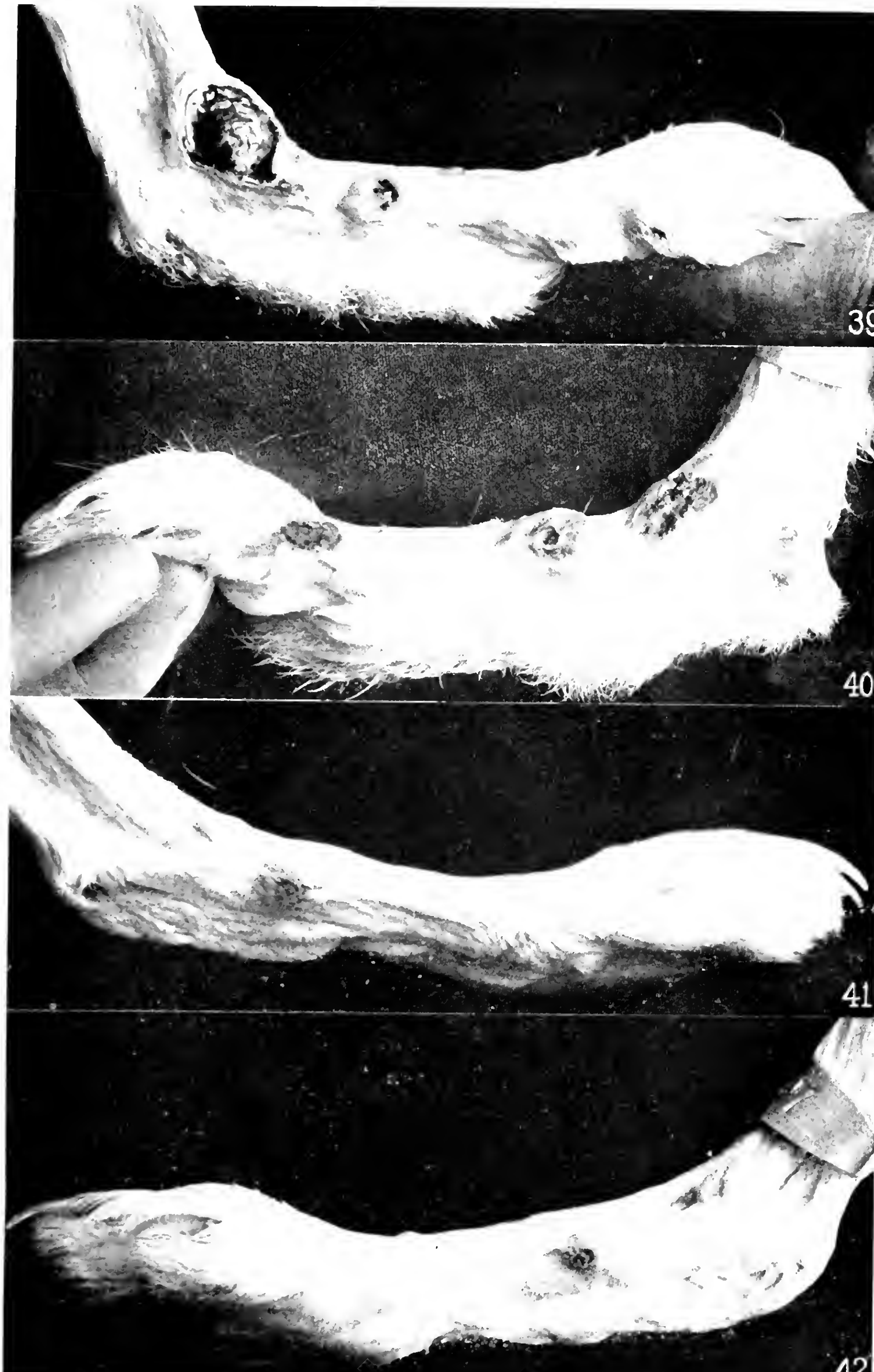


36

37

38

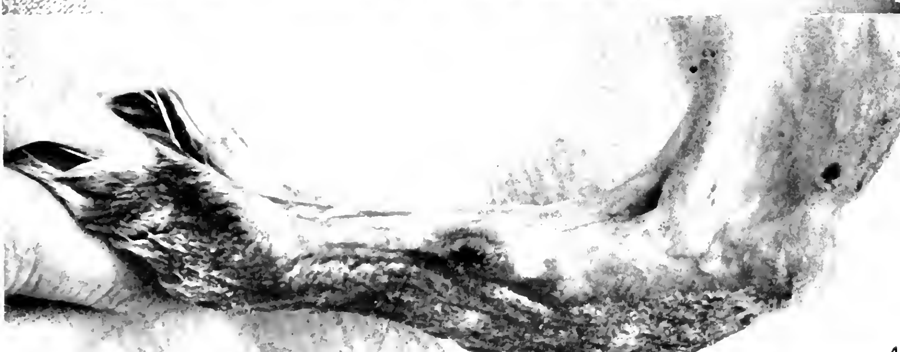
(Brown and Pearce: Experimental syphilis in the rabbit. IV.)



(Brown and Pearce: Experimental syphilis in the rabbit. IV.)

43

44



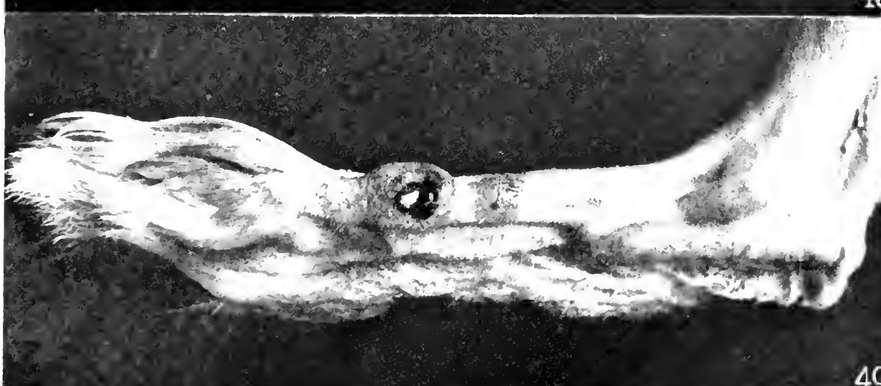
45

46

(Brown and Pearce: Experimental syphilis in the rabbit. IV.)

47

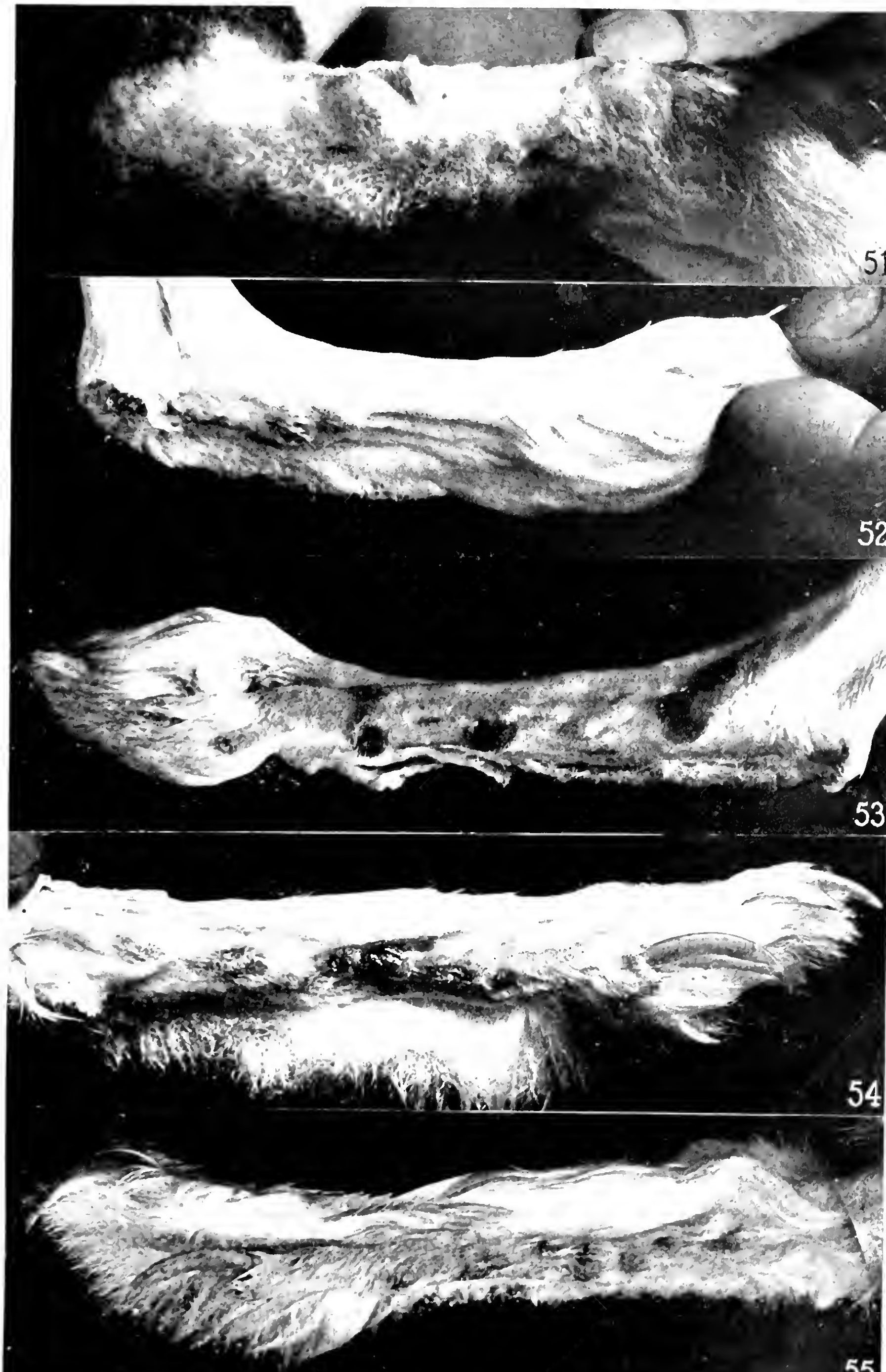
48



49

50

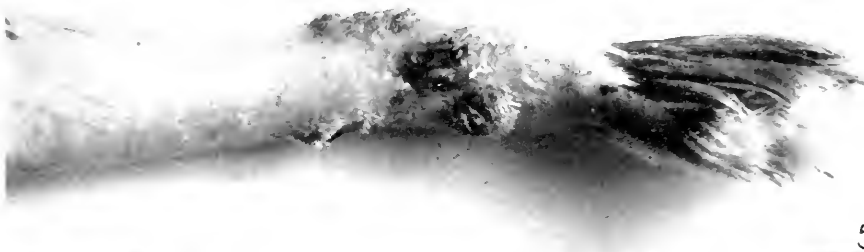
(Brown and Pearce: Experimental syphilis in the rabbit. IV.)



(Brown and Pearce: Experimental syphilis in the rabbit. IV.)

(Brown and Pearce: Experimental syphilis in the rabbit. IV.)

58



59

60

61

Brown and Pearce: [illegible] IV.

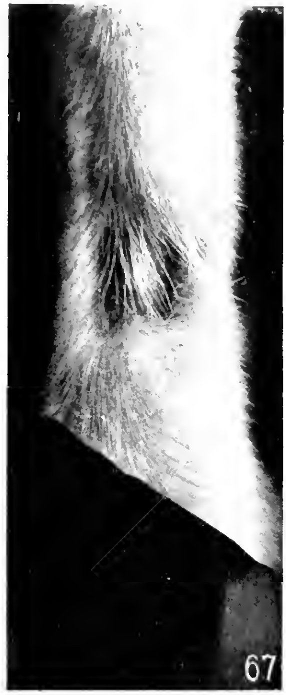
62

63

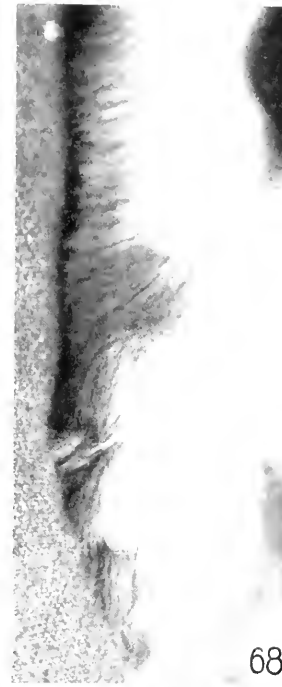
65

64

66



67

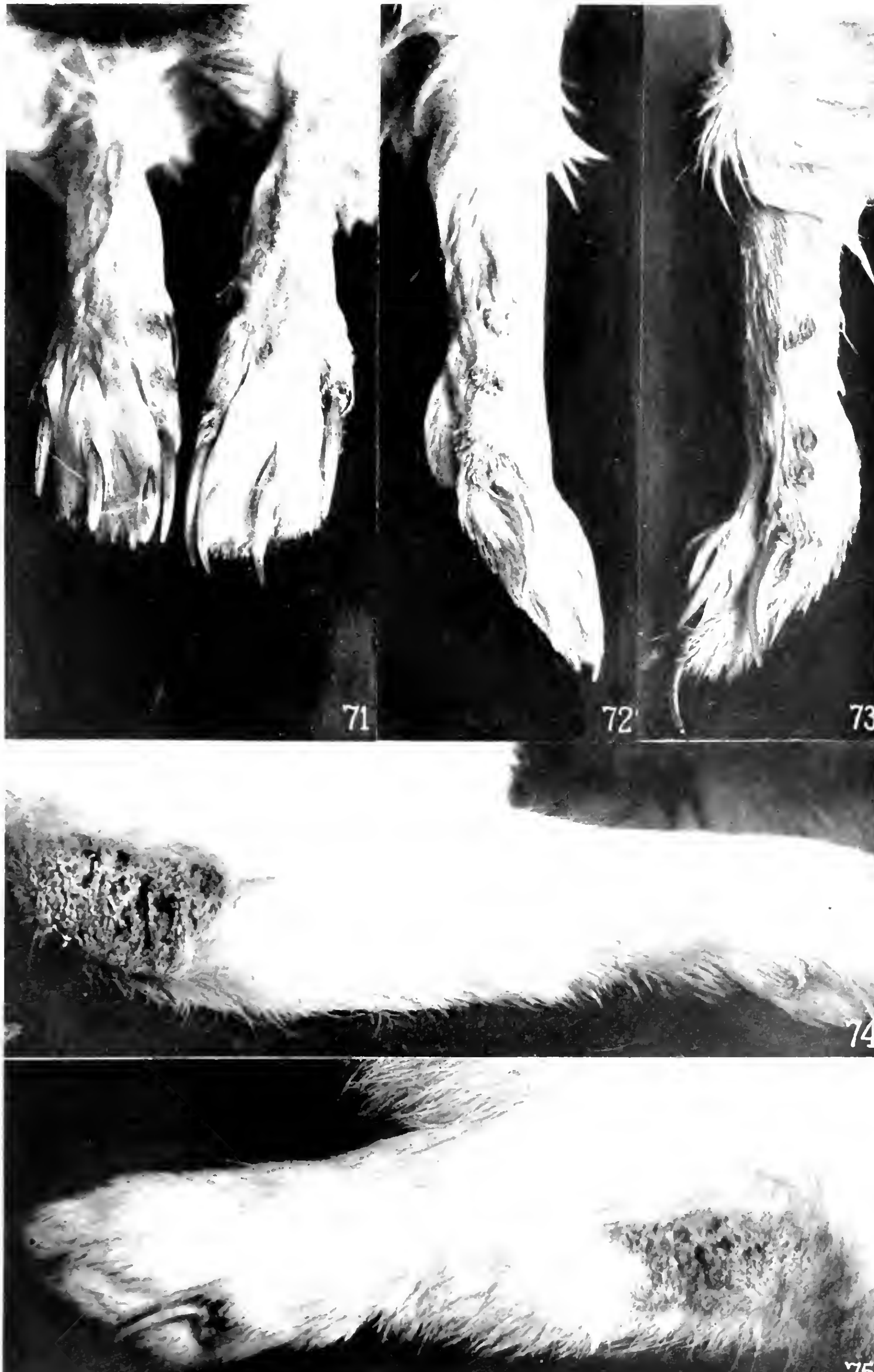


68

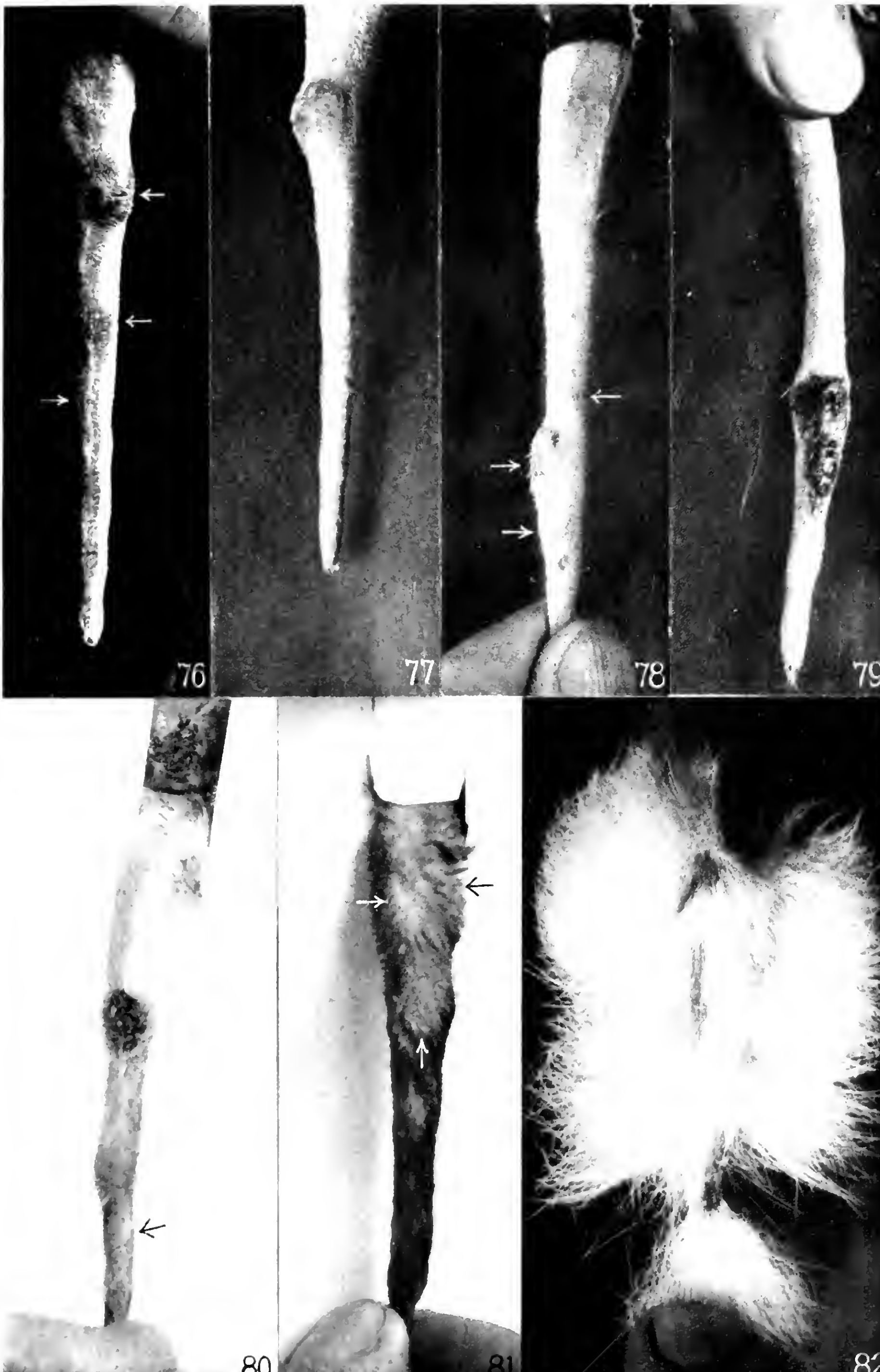
69

70

(Brown and Pearce: Experimental syphilis in the rabbit. IV.)



(Brown and Pearce: Experimental syphilis in the rabbit. IV.)



(Brown and Pearce: Experimental syphilis in the rabbit. IV.)

EXPERIMENTAL SYPHILIS IN THE RABBIT.

V. Syphilitic Affections of the Mucous Membranes and Mucocutaneous Borders.

By WADE H. BROWN, M.D., and LOUISE PEARCE, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 78 to 83.

(Received for publication, June 14, 1920.)

A study of cutaneous syphilis in the rabbit brought out the fact that although the infecting organisms might be widely distributed through the body, lesions of an easily recognizable character occurred almost exclusively within certain restricted areas while the remainder of the skin surface rarely showed any manifestation of disease (1, 2). In like manner, it has been found that lesions develop with considerable frequency in parts of the body where skin and mucous surfaces join. In some instances, the lesions first appear within the skin area, while in others they develop upon the mucous membrane so that it might be possible to classify most of them as affections of one or the other of these structures. It appears, however, that the transitional area exercises some influence upon the localization of the infection, and since lesions which develop in one tissue usually extend to the other, their classification as affections of mucocutaneous borders seems to be a more logical one.

Syphilitic involvement of the mucous membranes and mucocutaneous borders of the rabbit was first noted by Grouven (3), who described infiltrations of the nasal mucosa and rhagades about the nasal orifices, conjunctivitis, and papular lesions of the anus and sheath. Attention was also called to the presence of a nasal discharge containing spirochetes and to dyspnea presumably resulting from infiltration and swelling of the nasal mucosa. These early observations have been confirmed and amplified to some extent by subsequent observers, but no material additions have been made to the list of conditions originally described by Grouven. Uhlenhuth and Mulzer (4) described tumor-like swellings of the mucous membranes following generalized inoculations, and within recent

years, attention has been directed more particularly to the occurrence of diffuse inflammatory processes of the nose and sheath which show no characteristic lesions but are identified by a mucopurulent discharge which contains spirochetes. These observations constitute the chief contributions which have been made to the study of affections of the mucous membranes and mucocutaneous borders in the rabbit.

Among the animals first studied by us, localized infections of the mucous membranes and mucocutaneous borders were noted in about 20 per cent of the cases but have been less frequent among those studied more recently. The affections seen in these animals were of two general classes, depending upon the type of the lesions present. In one group, the lesions were characterized by diffuse infiltration, surface erosion or ulceration, and the formation of exudates of various types; in the other, there was a greater degree of proliferation and the lesions formed were large granulomatous masses which showed the usual secondary transformations of syphilitic processes. Affections of these two classes were distributed about the nares, the lips, the margins of the lids, the genitalia, and the anus.

Affections of the Nasolacrimal System and of the Nasolabial Region.

Infections of the nasolabial region were relatively infrequent, which, as will be explained later, may have been due to the fact that lesions in these areas were of comparatively late development and the animals were not held for a sufficiently long time.

The most common affection seen was a condition which in its earlier stages closely resembled an ordinary case of snuffles. The infection began as a rhinitis with a more or less profuse mucopurulent discharge and apparently extended to the nasolacrimal ducts, producing obstruction and consequent overflow of the lacrimal secretion.

At the onset of the local infection, no characteristic lesions could be detected by ordinary means of examination, but within a short time the discharge from the nose became of a more tenacious character and tended to adhere to the surfaces about the nasal orifices. There was then a noticeable reddening and swelling of the skin and mucous membranes, and removal of the accumulated discharge revealed the presence of minute abrasions as indicated in Fig. 1. An earlier stage of the process may also be seen in Fig. 9 which is a photograph of the same animal as that in Fig. 1, taken 1 month earlier.

As the local infection advanced, the skin areas became denuded of hair; the infiltration of the surrounding tissues was increased, especially along the edges of the alæ nasi, and the nares were obstructed by the presence of accumulated discharges or by thick adherent crusts, the removal of which left numerous raw and bleeding points (Figs. 1 and 2).

Several modifications of this general type of condition were seen. In some animals, the nasal discharge was comparatively slight, and the lesions were of a less diffuse character. Irregular areas of infiltration and rhagades occurred along the margins of the alæ and more especially at the angles of the nares. Occasionally also, simple areas of ulceration or ulcers surrounded by a definite zone of infiltration such as that in Fig. 3 were seen just at the edges of the nasal orifices.

Another very characteristic affection of the nasolabial region is that shown in Fig. 4. The condition as presented by this animal was a marked infiltration of the nasal mucosa and the tissues surrounding the left nasal orifice. The right side of the nose was also affected to a slight extent. The cutaneous portion of the lesion was covered by an accumulation of epithelial scales and crusts, while the mucous surface showed ulcers with a gray necrotic base.

An unusually destructive condition seen in one animal of this group is that shown in Figs. 5 to 8. The usual symptoms of acute rhinitis were absent in this case. When first detected a circumscribed, indurated mass was present in the superior portion of the right nostril and a smaller area in the left (Fig. 5). These lesions increased very rapidly, giving great breadth and prominence to the alæ (Figs. 5, 6, and 10). Depressed ulcers developed upon the mucous surfaces and spread until the skin margins were involved. Heavy reddish brown crusts were then formed and practically occluded the anterior nares on both sides (Fig. 7). The necrosis associated with these lesions was quite marked and led to considerable destruction of the soft tissues and consequent deformity of the nose (Fig. 8). At autopsy, it was found that a large part of the mucous membrane of both nasal passages had become involved and the left chamber was practically obliterated for a distance of about 2 cm. above the anterior orifice. Whether this was due to the *pallidum* infection or was the result of an associated bacterial infection could not be determined.

One other form of lesion was observed at the margins of the nares, and this also was seen in but a single animal. The lesion in this case was a discrete papule about 3 or 4 mm. in diameter situated on the lip near the median line. The tumor-like swellings of the nasal mucosa described by Uhlenhuth and Mulzer (4) were not observed in any of these animals. They have been noted by other observers (5), however, and it is possible that some of the conditions which we have described as infiltrations of the nasal mucosa may represent processes analogous to those referred to as nasal tumors.

Taken as a whole, this group of conditions presented a very characteristic appearance and resembled in many respects the nasal

affections of infants with hereditary syphilis. The essential feature of the lesions was an infiltration of the skin and mucous membranes with irregularly distributed areas of necrosis and ulceration, while the characteristic symptoms were the presence of a mucopurulent discharge, epiphora, or conjunctivitis, and dyspnea, the latter being apparently an obstructive phenomenon.

Lacrimation or lacrimal overflow has been mentioned by different writers as a symptom of infection of the nasal mucosa, and while there was a very constant association of these conditions, the causative connection between the two processes was not entirely certain. During the early stage of the nasal infection, the characteristic condition of the lacrimal system was that shown in Figs. 9 and 10. At this period, the cheeks below the anterior angle of the eye were bathed with a clear lacrimal secretion. The hair was matted together, the skin was slightly inflamed, and usually there was some loss of hair over these parts. The conjunctiva in some instances remained perfectly clear, while in others there were varying degrees of an acute inflammatory reaction. Eventually, the lacrimal secretion was altered, becoming more clouded, or mucopurulent, in character. The conjunctivæ were then dulled, and the secretion accumulated over the cheeks in greater amount as in Fig. 11.

Other workers have reported the presence of spirochetes in the lacrimal secretions, but repeated examination in these cases failed to show them. While this in itself is not conclusive, it suggests that these affections might be referable to occlusion of the nasolacrimal ducts either as a result of involvement of the nasal mucosa or of infection of the ducts themselves rather than to localized infection of the conjunctival sac. Except for its periodicity and its persistence, this feature of the nasal infection differed in no way from a similar condition frequently observed in rabbits from other causes.

Occurrence and Duration.—As has been stated, affections of the nasal region were not among the early manifestations of generalized syphilis. The majority of those seen were either late in their development or at least occurred subsequently to other types of lesions. The time at which they were first recognized varied from a minimum of 9 weeks to a maximum of 8 months after inoculation, but there were very few cases in which lesions appeared within the first 4 months.

These statements may give an erroneous impression as to the time at which the infection actually became localized in the mucous membranes of the nose or about the mucocutaneous margins, since the early

symptoms were in most cases but little more than an ordinary rhinitis and specific infection was recognized only after the condition had advanced sufficiently to arouse suspicion as to its etiology. For the same reasons, many cases of nasal infection may have passed unrecognized, since it was not possible to make routine examinations of all nasal discharges.

This group of lesions was not only late in developing but was also of a very enduring character. With a few exceptions, the animals of this group were held for a number of months after the development of the localized infection, and no instance was recorded of complete healing of the lesions during the period of observation. Two of the animals were held for approximately 1 year, and a third was under observation for more than 2 years. In the last animal, there were several periods during which the lesions about the nares underwent almost complete resolution, but each time there was a recurrence. It seems probable, therefore, that this group of conditions may be regarded as among the most persistent of the generalized infections in the rabbit.

Affections of the Lips and Buccal Cavity.

Syphilitic lesions were occasionally noted upon the mucous surfaces or along the margins of the lips, but the entire buccal cavity of the rabbit is an almost unexplored region. Small papular infiltrations were seen on the skin surfaces or at the mucocutaneous borders of both the upper and lower lips as described in the fourth paper of this series (1, 2), but even these were rare. In addition, lesions were found about the cleft in the upper lip or upon the mucous surfaces of the lips.

The region of the cleft in the upper lip was one of especial interest. Normally, the contact surfaces of this area are covered by a short downy growth of hair which reaches practically to the inner margins as shown in Fig. 12. There were several animals in which an infiltration about the nasal orifices continued downward along the margins of the nasolabial folds, forming a thickened ridge, the surface of which was bare and covered by scales or by gray necrotic patches and small moist areas of erosion. A condition of this kind was present on the left lip of the animal shown in Fig. 4.

In addition to such processes as these, there were a few instances in which independent affections of the cleft were observed. The lesions appeared in the form of small papules or flattened patches of infiltration which tended to spread over the surface of the lips, and the resulting affection presented a very characteristic appearance which is shown in Fig. 13. It will be noted here that the affected portions of the lips are moist and denuded of hair, that there is a distinct thickening of the left lip, and that the surfaces are marked by an irregular network of ridges covered by gray necrotic areas. Eventually small superficial ulcers were formed which bore a striking resemblance to the mucous lesions of man.

Similar affections were also noted on the mucous surfaces of the lips, but, as a rule, these were small erosions of an indifferent character in which no spirochetes could be demonstrated. In a single instance, however, an ulcer with definitely infiltrated margins was found upon the inner surface of the upper lip (Fig. 14).

Another type of condition which was seen in a number of rabbits consisted of small papillomatous growths which were occasionally present on the margins or inner surfaces of the lips, about the gums of the lower incisors, and were especially numerous on the sides and under surface of the tongue. While these affections resembled in some respects certain of the hypertrophic or vegetating lesions of man, we could obtain no proof of a syphilitic origin—either clinical or histological.

Occurrence and Duration.—Affections of the lips were in part no more than extensions from those about the nose and were subject to the same general conditions. The independent affections of the lips appeared to be of earlier development and of comparatively short duration. There were very few of these, however, and too much cannot be inferred from such a small group of cases.

Affections of the Eyelids.

The lesions seen on the eyelids might be separated into two distinct classes according to their location, those which apparently originated in the skin and seemed to bear no particular relation to the marginal area, and those which originated at the mucocutaneous junction of the lid or ultimately involved this area. The first group of conditions was described in the paper dealing with cutaneous lesions. The affections of the mucocutaneous borders proper were also of two general types, first, papular or granulomatous lesions (Figs. 15 and 16), and second, lesions which appeared in the form of ulcers along the margins of the lids (Fig. 17).

The papular and granulomatous lesions presented no essential difference from the skin affections previously described, except as they came to involve the conjunctival surfaces. When this occurred, there was usually a moderate degree of conjunctivitis with reddening and swelling of the conjunctiva and increased lacrimation. These symptoms, however, were only transient, and within a few days, such inflammatory reactions were confined to the immediate area of the lesion (Fig. 16).

Lesions which originated upon the margins of the lids or on the surface of the conjunctiva itself usually produced a greater degree of inflammatory reaction. In most instances, they appeared, as we have said, in the form of small ulcers or abrasions. Occasionally, the initial affection was a minute papule, such as that in Fig. 15, which subsequently underwent ulceration. The appearance presented in these cases depended largely upon the extent of the induration which was associated with the formation of the ulcer. In several instances, lesions of this type developed definite collars of induration such as that shown in Fig. 17. As will be seen from this figure, there was a well marked area of infiltration extending from the margin of the lid back under the surface of the conjunctiva.

Occurrence and Duration.—In the few animals which showed lesions upon the margins of the lid, the time of appearance of these lesions varied from about 3 to 11 months after inoculation, but the majority occurred within 4 months. On the whole, they were decidedly less enduring than those about the nose, but in exceptional instances, they lasted for several months with varying periods of activity and quiescence or regression.

Affections of the Penis and Sheath.

As in the case of localized infections about the nose, it is probable that many cases of specific infection of the penis and sheath occurred which did not lead to the formation of lesions of a sufficiently definite character to attract our attention. Among the animals in this group, there were several in which the diagnosis of the local infection was made prior to the development of any characteristic lesion. These cases were suspected on account of redness and some swelling of the sheath and the presence of a mucopurulent discharge which upon examination was found to contain *Treponema pallidum.* Otherwise the diagnosis was first made on account of the presence of lesions which proved to be syphilitic.

The list of affections which occurred about the penis and sheath included first, cases of a diffuse inflammatory character associated with a mucopurulent exudate, second, circumscribed or diffuse infiltrations which usually led to the formation of papules or ulcers, and third, lesions of a granulomatous type consisting either of circumscribed masses or of more diffuse granulomatous processes.

The acute inflammatory process which marked the beginning of many of these localized infections was a fairly characteristic feature of affections of the moist surfaces or the orifice of the sheath. In one group of cases, there was a diffuse redness and swelling of the parts as in Fig. 18, which lasted for only a short time as a rule and was superseded by a diffuse infiltration (Fig. 19). As the process advanced, erosions or definite ulcers made their appearance in various localities but more especially about the margins of the sheath. Lesions on the skin surface were covered by dry, adherent crusts, while those on the mucous surfaces of the sheath and the shaft of the penis were moist and covered by a gray necrotic exudate. Occasionally small papules or condylomatous growths were also present (Fig. 18).

In another group of cases, the initial lesion was of a more circumscribed character and appeared in the form of a slightly elevated area of a gray, pink, or amber color surrounded by a zone of slight inflammatory reaction (Figs. 20 and 22). Some of these processes tended to spread and formed diffuse areas of infiltration (Figs. 20 and 21), while others developed into circumscribed and indurated nodules as was the case with the lesion shown in Figs. 22 and 23.

When the infection was confined to the skin surface, the acute inflammatory reaction was usually slight and the conditions present were much the same as in the case of other skin lesions. Thus in Fig. 24 there is seen an area of infiltration involving the right side of the sheath which is just beginning to show surface necrosis and eventually developed into the chancre-like mass seen in Fig. 25.

This type of affection was comparatively common and presented numerous variations of the condition illustrated. Two especially marked cases of infection confined to the region of the meatus of the sheath are shown in Figs. 26 and 27. It will be seen that the lesions completely surround the orifice of the sheath and are located practically at the line of junction of the skin and mucous surfaces. In one case (Fig. 26), the necrosis appeared exactly along this line and completely encircled the meatus. In the other (Fig. 27), the necrotic area occupied a similar position, but at the time the photograph was taken, the ulcer was confined to one side.

Another form of unusually marked involvement of the sheath is that shown in Figs. 28 and 29. The condition began with edema and congestion of the sheath, and, as the lesions developed, there was marked enlargement and induration of the sheath extending from meatus to base, associated with a pronounced exfoliation of surface epithelium and the formation of cracks and erosions of the skin

and mucous surfaces (Fig. 28). Eventually, an irregular line of necrosis and ulceration appeared and separated the mucous from the skin surfaces. On the whole, the mucous membrane appeared to be less involved than the skin, and no definite involvement of the penis could be made out.

Involvement of the penis, as has been mentioned, may occur along with that of the mucous surface of the sheath, in which case the lesions seen were of the type of small infiltrations or surface erosions. One other form of lesion may be mentioned which was observed in but one animal. This consisted of an elevated area of infiltration surrounding the urethral orifice as is indistinctly shown in Fig. 30. In addition, it may not be out of place to mention that infection of the mucous membrane of the urethra with the production of lesions has been demonstrated microscopically.

Occurrence and Duration.—As might be expected from their proximity to the point of inoculation, lesions of the sheath developed somewhat earlier than those of the other mucocutaneous surfaces. The earliest lesions of this group appeared 26 days after inoculation, in other words, almost as soon as the primary lesion itself. There were two other cases which occurred within 2 months after inoculation, and a number of cases within a period of 3 months, while the latest case of the series was 6 months after inoculation. The subsequent history of lesions of this group was very variable. Some lasted for a comparatively short time, while others endured for many months. For example, the lesions shown in Figs. 28 and 29 were still fairly active when the animal was killed for pathological examination $5\frac{1}{2}$ months after the lesions first appeared, and in a second animal, the lesions persisted for a little more than a year.

Affections of the Anal Region.

With a few exceptions, animals which showed specific involvement of the sheath showed lesions of the anus as well, and, conversely, there were only two animals with anal lesions in which corresponding lesions were not present on the sheath. The two groups of conditions, therefore, might almost be considered as one, and apparently the main difference between them was in the character of the lesion which occurs in the two locations, and even this was not great. There were no true exudative affections of the anus. The lesions situated on the cutaneous surfaces were essentially the same in all respects as those

of the sheath. In fact, there was a very striking similarity between the lesions present in the two localities, as may be seen by comparing those shown in Figs. 24 to 29.

The most interesting conditions seen by us in the anal region of the rabbit were lesions which might be classed as condylomata, a typical example of which is given in Fig. 31. These lesions were, as a rule, entirely obscured from observation until the anal ring was distended to a sufficient extent to permit of inspection of the transitional borders. This could be easily done by exerting pressure upon the rectum immediately behind the sphincter.

The first suggestions of the presence of lesions of this character (condylomata) came with slight redness and swelling of the anus, and during the earlier stages of the localized infection, no other alteration might be present. As infection advanced, the reaction became more localized, with the development of areas of infiltration and induration situated in one or more segments of the anal ring immediately along the junction of the skin and the mucous membrane. These were not unlike the patches of infiltration on the mucous surfaces of the sheath. In cases of more pronounced involvement, however, such as that shown in Fig. 31, the entire anal ring was involved, with extension of the process over both the mucous and cutaneous surfaces. The lesion thus formed was a distinctly elevated and indurated mass strikingly like the condyloma latum in man. Judging from our small series of animals, lesions of the condyloma type are more frequent about the anus of the rabbit than on the sheath, and this constituted the chief difference between the lesions of the two localities.

Occurrence and Duration.—As regards the time of occurrence and duration of anal lesions, they are again comparable to those of the sheath, but, on the whole, appeared to be slightly more delayed in their development and in a few instances were more enduring. The condyloma shown in Fig. 31 remained active for upwards of 15 months and still showed slight signs of activity when the animal was killed 21 months after inoculation.

Diagnosis of Mucocutaneous Infections.

Obviously there are many conditions affecting the rabbit which might be difficult to differentiate clinically from some of the affections described above. This is especially true of infections of the nose, eyes, and genitalia, but rare in the case of the anus. A syphilitic rhinitis which is not associated with characteristic lesions presents much the same train of symptoms as the more common condition

known as snuffles. The chief symptomatic difference between the two affections is in the associated involvement of the lacrimal system. As a rule, snuffles is not associated with marked and persistent lacrimal overflow, and when involvement of the lacrimal system does occur, there is usually an acute conjunctivitis. On the other hand, profuse lacrimation without an acute inflammatory reaction is very common even in the early stages of a syphilitic infection. At this stage, however, the distinction between the two processes can be made with certainty only by a demonstration of spirochetes in the nasal discharge or in the mucous membranes themselves.

In cases in which lesions are present, the differentiation of the two conditions presents less difficulty. In the most severe and long standing cases of snuffles, some infiltration and erosion may be present about the nares but they are associated with a greater degree of suppuration and could hardly be confused with the conditions described above.

The two main conditions to be considered in connection with the eyelids and conjunctiva are trauma and conjunctivitis of bacterial origin. Slight abrasions of the lids are fairly common among rabbits, especially as a result of scratching, but they usually heal very quickly and should not be confused with syphilitic infiltrations. There is also a parasitic disease of the skin (mange) which occasionally forms small lesions along the margins of the lids, but these are readily identified by the character of the lesion and the presence of a similar affection upon other parts of the body.

Traumatic conjunctivitis and epizootic infections of the conjunctiva must also be distinguished from affections of a syphilitic nature. These conditions, however, have a more immediate connection with affections of the eyes than with those of the present group.

There are several conditions, chiefly infections, which should be mentioned as possible sources of confusion with syphilitic involvement of the penis and sheath. So called gleet is a well known disease of rabbits which affects the genitalia of both males and females and is characterized by an acute inflammatory reaction with a purulent exudate and may lead to necrosis and ulceration. We have seen a few such cases among normal animals but know very little of this condition from personal observation.

Arzt and Kerl (6) have described a similar condition in rabbits due to a spirochete infection which is capable of transmission from one animal to another. It is claimed that the lesions produced by this organism bear some resemblance to syphilitic lesions. We have never encountered infections of this type.

Traumatic and pyogenic infection about the genitalia must also be considered, but as far as we are aware, the only class of syphilitic affections which might be confused with any of the conditions mentioned are those in which no characteristic lesions are present, and in these cases, a diagnosis can be made only by the demonstration of spirochetes.

SUMMARY.

In a series of more than 200 rabbits in which generalized lesions were observed following local inoculation with *Treponema pallidum*, there were a number of animals in which characteristic lesions were noted upon mucous membranes or along mucocutaneous borders. These lesions were distributed with about equal frequency between the nose or nasolacrimal system and the eyelids on the one hand, and the genital and anal regions on the other. The lips and buccal mucosa appeared to be less subject to localized infections unless the papillomatous growths noted on the lips and under side of the tongue should prove to be in some way connected with such an infection.

In many instances, the local reaction was initiated by an acute inflammatory process, and in the case of nasal and genital infections, a definite exudate was formed. The succeeding stages of the reaction consisted in an infiltration of the parts involved, together with a variable degree of proliferation of fixed tissue cells, leading eventually to necrosis and ulceration. The resulting lesions differed according to their location and the character of the reaction in the individual case. Localized infections of the nose occurred in several forms, first, as a rather diffuse affection of the nasal mucosa characterized by the presence of a mucopurulent exudate, second, as a more or less circumscribed process of infiltration with an especial predilection for the region of the anterior nares, and third, as a granulomatous process involving the alæ in particular.

Involvement of the nasal mucosa was very commonly associated with lacrimal overflow and with some degree of conjunctivitis.

The lesions of the eyelids were usually small, elevated papules or lesions of an ulcerative character some of which were surrounded by a zone of infiltration. In exceptional instances, large granulomatous lesions occurred along the margins of the lower lids.

Infection of the penis and sheath gave rise to conditions analogous to those of the nose. In one group of animals, there was a diffuse affection characterized by redness and swelling of the parts with a mucopurulent exudate, in another there were circumscribed or diffuse infiltrations, while in a third the lesions formed were indurated granulomatous masses. Secondary necrosis with erosion or ulceration was a common feature of all these conditions.

Localized infections in the region of the anus differed from those in other localities chiefly in the absence of an exudative group of affections and in the frequency with which lesions of a papillomatous type occurred.

Lesions of mucous membranes and mucocutaneous borders developed at periods of time varying from a few weeks to several months after inoculation. Most of them were rather enduring and in several instances persisted in an active condition for considerably more than a year.

BIBLIOGRAPHY.

1. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1920, xxxii, 445.
2. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1920, xxxii, 473.
3. Grouven, *Dermat. Z.*, 1908, xv, 209.
4. Uhlenhuth and Mulzer, *Arb. k. Gsndhtsamte*, 1910, xxxiv, 222.
5. Reasoner, *J. Am. Med. Assn.*, 1916, lxvii, 1799.
6. Arzt, L., and Kerl, W., *Wien. klin. Woch.*, 1914, xxvii, 1053.

EXPLANATION OF PLATES.

The illustrations are reproductions of unretouched photographs which, with the exception of Fig. 3, represent the objects at their natural size. Fig. 3 is at a magnification of 1.5. Statements of time are estimated from the date of inoculation unless otherwise indicated.

Plate 78.

Figs. 1 to 4. Syphilitic affections of the nasolabial region.

Fig. 1. 4 months. Syphilitic rhinitis with infiltration about the nares. There is loss of hair over the affected area, with superficial necrosis and ulceration and the formation of adherent crusts.

Fig. 2. 10 months. Marked infiltration of the alæ with necrosis and the formation of ulcers covered by crusts. There were also pronounced lesions at the angles of the nares.

Fig. 3. 68 days. A circumscribed and elevated area of infiltration with central necrosis and ulceration situated in the right nasal orifice. The lip is drawn down to bring the lesion into view.

Fig. 4. 5½ months. A marked diffuse infiltration of the skin and mucous membrane of the left side of the nose and the nasolabial fold with slight involvement on the right. The skin area shows exfoliation of surface epithelium and there were erosions on the mucous membrane.

Plate 79.

Figs. 5 to 8. Stages in the progress of an ulcerating granulomatous lesion of the nose.

Fig. 5. 4½ months. Early granulomatous lesions in the anterior nares with an ulcer on the right. There was no nasal discharge at this time.

Fig. 6. 2 weeks later. A slightly later stage of the affection showing the marked prominence of the alæ and the extension of the necrosis and ulceration, now present on both sides.

Fig. 7. 7 months after the appearance of the lesions. There has been marked destruction of the soft tissues of the nose, and both nostrils are occluded by thick reddish brown crusts.

Fig. 8. The same lesions 1 week later with the crusts removed. There was still slight activity in some parts of the lesions most noticeable on the right. The left nasal orifice was entirely obliterated, and there was only a small opening on the right.

Plate 80.

Figs. 9 to 11. The clinical appearance presented by animals showing a combined nasolacrimal involvement.

Fig. 9. 3 months. The same animal as that in Fig. 1, showing an earlier stage of the affection. Note the characteristic mucopurulent discharge about the nose associated with lacrimal overflow. This condition is not unlike that sometimes seen in snuffles.

FIG. 10. 5 months. The same animal as that in Figs. 5 to 8. A pronounced lacrimal overflow was present in this animal without an associated nasal discharge. Note the swollen condition of the lids and skin at the anterior or internal angle of the eye.

FIG. 11. The same animal at a later period of the infection (1 year after inoculation). Chronic dacryocystitis.

PLATE 81.

FIGS. 12 to 14. Affections of the labial cleft.

FIG. 12. The appearance of the surface of the labial cleft in a normal rabbit.

FIG. 13. 3 months. A diffuse infiltration of the skin and adjacent mucous surfaces of the labial cleft, more marked on the left than on the right. The skin is denuded of hair on both sides, and the left lip shows a series of irregular ridges covered by gray necrotic epithelium. Mucous patches.

FIG. 14. $3\frac{1}{2}$ months. A sharply circumscribed and indurated ulcer on the inner surface of the left upper lip.

FIGS. 15 to 17. Lesions of the margins of the lids.

FIG. 15. $2\frac{1}{2}$ months. An early papular lesion arising in the margin of the lower lid.

FIG. 16. 3 months. The lower lid everted to show the inflammatory reaction on the conjunctival surface resulting from a syphilitic lesion on the lid.

FIG. 17. 6 months. An indurated ulcer on the upper lid and congestion of the conjunctival vessels.

PLATE 82.

FIGS. 18 to 25. Affections of the penis, sheath, and anus.

FIG. 18. $4\frac{1}{2}$ months. Diffuse infiltration of the sheath with edema and congestion of both the penis and sheath. There are small papular lesions on the sheath and an area of erosion at the superior margin of the fold in the sheath.

FIG. 19. 145 days. A later stage of the lesions in Fig. 18 showing diffuse infiltration and thickening of the sheath.

FIG. 20. 47 days. A small circumscribed area of infiltration on the mucous surface of the sheath with a diffuse redness and swelling. Sheath retracted.

FIG. 21. 2 weeks later. The same animal. The infiltration has become more pronounced and retraction of the sheath is difficult. Congestion and edema have subsided.

FIG. 22. $2\frac{1}{2}$ months. An early papule situated in the transitional area between the skin and mucous surfaces of the sheath. The papule is surrounded by a slight zone of acute inflammatory reaction.

FIG. 23. 2 weeks later. The same animal. The lesion on the sheath has developed into an indurated nodule with central necrosis and ulceration. The edges of the ulcer are inverted and there are marked vascularization and redness of the tissues at its base (mucous surface).

FIG. 24. 3 months. An irregular area of infiltration in the skin of the sheath. The surface of the lesion shows beginning necrosis at two points. On the dorsal surface of the anus, there is also an indurated granulomatous lesion with a depressed ulcer at the center.

FIG. 25. 1 week later. The same animal. Both lesions have increased somewhat and are now of essentially the same character.

PLATE 83.

FIGS. 26 to 31. Affections of the penis, sheath, and anus.

FIG. 26. 3 months. Syphilitic lesions of the anus and sheath situated at the mucocutaneous borders and completely encircling these parts. The lesions were characterized by an intense induration and the development of a line of necrosis which practically coincided with the transitional area.

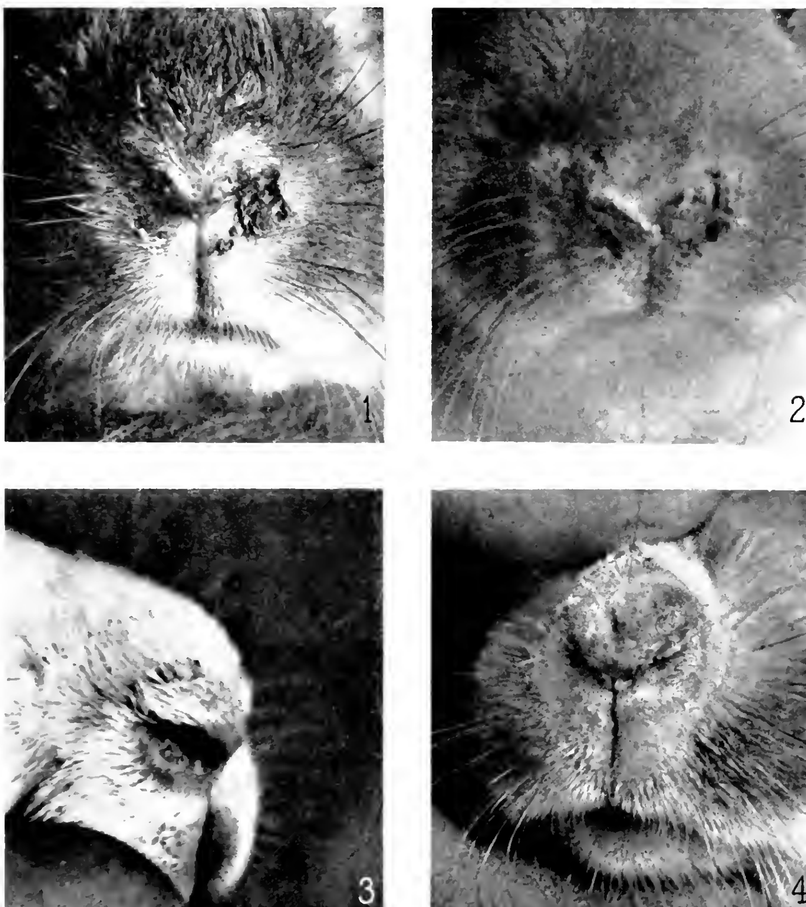
FIG. 27. 3 months. A similar group of lesions in another animal. The lesion on the anus, however, was confined to one side.

FIG. 28. 3 months. A diffuse infiltrative process involving the entire sheath and anal ring. The surface of the lesion was marked by irregular areas of superficial necrosis and exfoliation; there were also erosions on the mucous membranes.

FIG. 29. 1 month later. The same animal. Note the appearance of a line of necrosis separating the swollen and everted mucous membrane from the skin of the sheath. There was a similar condition of the anal ring.

FIG. 30. 2 months. A small mass is seen on the under surface of the penis (marked by an arrow), which was formed by a zone of infiltration surrounding the meatus of the urethra.

FIG. 31. 6 months. Condyloma latum of the anus. The anus is everted.



(Brown and Pearce: Experimental syphilis in the rabbit. V.)

(Brown and Pearce: Experimental syphilis in the rabbit. V.)

11

10

9

(Brown and Pearce: Experimental syphilis in the rabbit. V.)

12

13

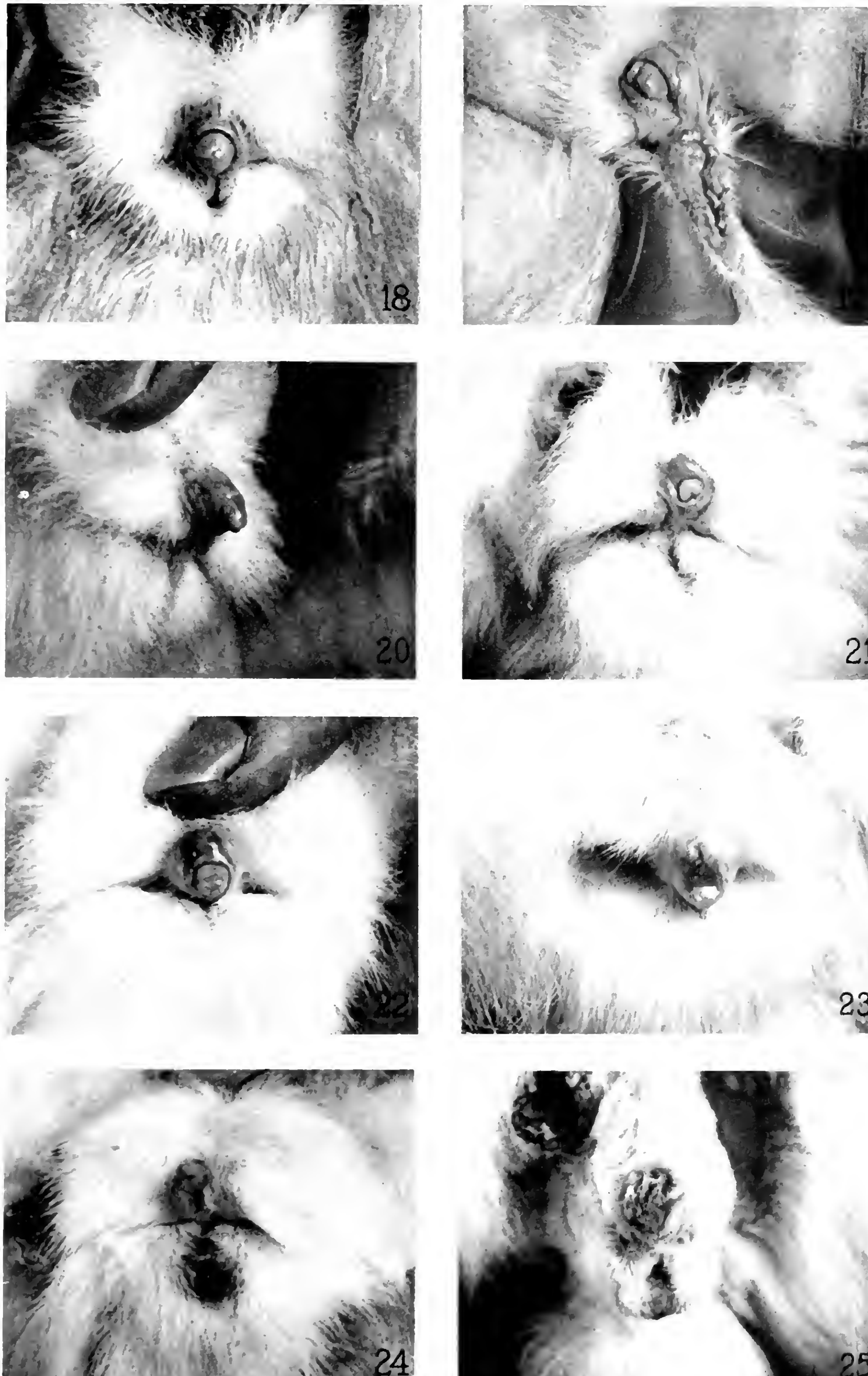
14

15

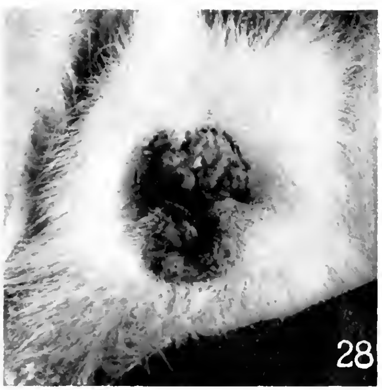
16

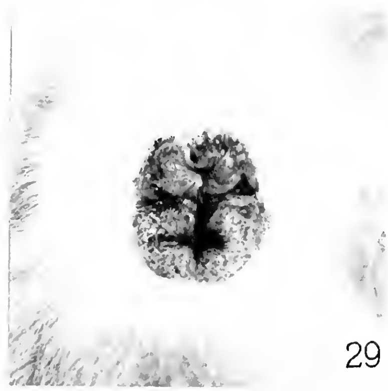
17

(Brown and Pearce: Experimental syphilis in the rabbit. V.)



Brown and Pearce: Experimental syphilis in the rabbit. V.





(Brown and Pearce: Experimental syphilis in the rabbit. V.)

STUDIES ON ENDOTHELIAL REACTIONS.

II. The Endothelial Cell in Experimental Tuberculosis.

By NATHAN CHANDLER FOOT, M.D.

(*From the Department of Comparative Pathology, George Fabyan Foundation, Harvard Medical School, Boston.*)

Plates 84 to 86.

(Received for publication, May 5, 1920.)

In the first paper of this series (Foot, 1919) it was shown that the macrophages of the loose connective tissue of the rabbit are of endothelial origin, that they are not derived from the cells of the omentum, the fibroblast, or the lymphocyte, that they are probably chiefly produced by the proliferation of the vascular endothelium in the immediate vicinity of the lesion which calls them forth, and, lastly, that they do not appear to come from distant parts of the body by way of the blood stream, as has been suggested by some authors. The means employed in tracing them to their source was a lampblack gelatin colloidal suspension, or sol, prepared according to the formula of McJunkin and injected intravenously; the agent used to call them forth was sterile melted agar injected subcutaneously.

After studying the effect of so inert and simple an excitant as this, it is natural to turn one's attention to the more complex conditions brought about by microorganisms which are known or believed to cause a somewhat similar reaction, limited chiefly to the endothelium, but probably more variable than that already studied. For this purpose the reaction to the tubercle bacillus was first chosen; that to other organisms will be the subject of future study.

There has been much dispute concerning the origin of the component cells of the tubercle for many years. The epithelioid cell is said by some to be of endothelial origin, by others of purely connective tissue or fibroblastic parentage, while a third group of investigators traces them to a lymphocytic source. There are also some authorities who would admit epithelial and mesothelial cells to this category

of antecedent cell, feeling that the tubercle represents no one cell group alone, but rather a composite structure. This phase of the subject is discussed at length below. For the present we must consider whether the question can be answered by means of intravital staining.

The work of Goldmann, and Evans, Bowman, and Winternitz has shown it to be possible in the case of the liver, where they have demonstrated that the tubercles are formed by the proliferation of the Kupffer cells of the sinusoidal endothelium, using benzidine dyes to stain these cells intravitally and then producing tubercles by injecting tubercle bacilli directly into the portal system. The tubercles were vitally stained in every case. Oppenheimer obtained similar results in 1908, using collargol as the coloring matter.

It seems, therefore, that the origin of the tubercle from the endothelium is proved in the case of the liver. Kupffer cells, however, take up benzidine dyes very well, whereas the endothelium of the smaller capillaries elsewhere takes up little or none, so that it is necessary to choose a more specifically endothelial vital stain before it can be decided whether the tubercles of other organs are also of endothelial origin. It is the more important to decide this question since Sewell, using vital staining methods, has recently claimed that the alveolar epithelium is the chief source of the epithelioid cells of the pulmonary tubercle. Intravenous injections of a suspension of carbon, which must be in the colloidal form, will, as McJunkin (1918, 1919) has shown, serve as a vital stain nearly, if not quite specific for the entire endothelium of the smaller capillaries, particularly if they are subjected to inflammation or injury. It seems advisable, therefore, to produce tubercles in various parts of the body, and, by means of a combination of two or more of these vital stains, to mark the endothelial cell so as to trace it, if possible, to the lesions thus formed.

Before describing the work which I have done along these lines and the results obtained, it would be well critically to consider the specificity of a carbon suspension for the endothelium. It was found that if 5 cc. of a colloidal suspension of lampblack and gelatin, or of equal parts of Higgins' water-proof India ink and distilled water, in which acacia replaces the gelatin, are injected into the ear vein of a rabbit, the veins of the opposite ear become black in about 7 seconds; in a minute's time they resume their normal red color and stay red

until another injection is given. If the animal is killed after 45 minutes, the spleen, liver, and bone marrow are found to be almost black and the lungs definitely sooty; the spleen oozes ink on pressure. Microscopically the spleen shows much carbon free in the sinuses, but comparatively little is intracellular; later it is found to be taken up by the pulp cells, and other endothelial cells. The Kupffer cells of the liver are completely engorged with carbon after 45 minutes, their stellate processes being outlined by the particles of pigment. In the lungs many of the endothelial cells of the alveolar capillaries are filled with carbon and are seen to migrate into the air sacs. The kidney may show some carbon in the endothelium of the glomerular tufts, but none in the epithelium. A large percentage of mononuclear cells in the circulating blood contains the pigment, as may be demonstrated in films or in cross-sections of the larger vessels in various organs. A small amount of free pigment is found in the blood, but this disappears after 24 hours. McJunkin has found that the mononuclear cells of the circulating blood require from $\frac{1}{2}$ to 2 hours to become saturated with lampblack, and he has demonstrated that such cells will phagocytose carbon in large quantities *in vitro* within an hour, both in the case of animal and human blood. This author states that, after injection subcutaneously, no carbon is taken up by the fixed cells of the connective tissue within 2 hours, a fact which he thinks would prove that the phagocytic cells are not of connective tissue origin.

Experimental Subcutaneous Tuberculosis.

In order to test the validity of this seemingly sweeping claim and at the same time to study the earliest reactions to the tubercle bacilli when injected subcutaneously, three rabbits were thus injected with a mixture of tubercle bacilli and Higgins' ink, diluted with equal parts of distilled water, and the resulting lesions studied. It will be seen that the experiment was so arranged as to give a very even series of reactions ranging from 1 to 30 hours. In this way it is possible to follow not only the process of phagocytosis, but also the early reaction to the tubercle bacillus.

Experiment 1.—Three rabbits were given injections at hourly intervals so that a series of lesions would be obtained representing a lapse of from 1 to 30

hours. The injections were made into the subcutaneous tissue of the back, starting at the nape of the neck and continuing toward the tail. They were charted and the lesions numbered for identification.

The amount injected each time was 1 cc. of a mixture consisting of 10 cc. of normal salt solution, two loops of a culture of bovine tubercle bacilli on glycerol agar, and ten drops of Higgins' ink. This combination makes a very even colloidal suspension and gives results perfectly analogous to those obtained with lampblack gelatin. The preparation and sterilization of the latter are thus avoided, as there is enough camphor present to keep the ink sterile. The bacilli were removed from the culture tube and rubbed up in a drop or two of the salt solution with a flat platinum spatula until a cream resulted. Further comminution was obtained by bending the spatula into a J and spinning it rapidly in the cream with the thumb and forefinger. The resulting "mixture" was then diluted up to 10 cc., the ink added, and the whole was shaken for ½ hour in a mechanical shaker, the agitation being repeated before each injection. In this way a reasonably good suspension of the bacilli was obtained. After each animal had had nine or ten injections it was killed by forcing Zenker's fluid into the beating heart under an anesthetic, a large syringe and needle being used for the purpose. The animal was skinned, the lesions were dissected out and spread on paper, and then each lesion was dropped into a separate bottle of Zenker's fluid and fixed for 12 hours.

The material was embedded in paraffin and cut as thin as possible; it cut best when the sections were made at right angles to the flat surface of the lesion; those cut parallel with it were found to be unsuitable for very thin sections, but it was possible to make fair preparations of 10 to 15 microns in thickness, which was always done. They were stained by various methods: Mallory's eosin-methylene blue, phosphotungstic hematoxylin, and connective tissue stain; Van Gieson's connective tissue stain; and various combinations of hematoxylin and carbolfuchsin.

Lesions were obtained chiefly in the subcutaneous tissues, but several were made a trifle deeper into the muscle, and in one case a lymph node was included in the experimental field, the injection missing it by only a millimeter or so.

In describing the results of the experiment, no attempt will be made to itemize the lesions by hours, as it would be unnecessary. An outline of the changes noted will be given with ample indications of the time factors involved in each.

Changes Found after 1 to 7 Hours.—The first change wrought at the site of the injection is an extensive destruction of the subcutaneous tissue, which is forced apart and the cells of which are separated and badly damaged both mechanically and by the action of the large number of bacilli introduced; at first the mechanical injury pre-

dominates. The ink is distributed in a clinging layer over the coarser fibers and on the surface of the cells present, and remains extracellular for a time. Within the 1st hour little if any of it is taken up by the macrophages and fibroblasts, but after 2 hours it can be found within the cytoplasm and surrounding the nuclei of both of these. In the latter, however, it seems to be taken up in a finer form, and to a much less extent, as though these cells were incapable, or had lost their power of phagocytosing coarser particles; in the macrophages it is in a coarser form. Polymorphonuclear leucocytes appear in large numbers at the end of 2 hours and are found throughout the succeeding stages of the experiment. It is noteworthy that one can find an almost immediate response in the endothelium of the smallest capillaries; at the end of 1 hour the cells of their walls are swollen and increased in numbers, in some cases almost blocking the lumina of the vessels. Near these vessels are found a few migrating mononuclear cells of the type described in the first paper, ovoid cells with bean-shaped nuclei and fairly dense cytoplasm; after 5 hours they are not only markedly increased in numbers, but they are actively phagocytic for the carbon (Figs. 3 and 4). Before 4 hours time they are relatively hard to find, except in the immediate vicinity of the vessels, which are never abundant in the sections; after 5 hours one has no difficulty in observing these cells, and they are fairly evenly distributed through the sections. It is of significance that they should be first found near vessels only. The macrophages, or larger type of these wandering endothelial cells, now show definite degeneration, as do the fibroblasts. By far the largest number of healthy cells are those which have migrated into the field of injury, most of the preexisting tissue cells having become necrotic or at least degenerated.

Changes Found after 8 to 30 Hours.—The process continues up to about 8 hours, when it is noted that the small endothelial wandering cells begin to increase in size from 4.8 to 6.5 microns to from 9.5 to 16 microns. They contain carbon and the eosinophil granules of necrotic polymorphonuclears, as well as vacuoles. Some are of irregular, more or less globose shape, some are fusiform, others are stellate. At this time cells resembling fibroblasts begin to be conspicuous (Fig. 5) whereas heretofore they have been an almost negligible factor; despite this fact it is only after much searching that a very

few mitotic figures can be found in them. They usually contain carbon in an evenly divided, rather fine form. It is also noticeable that, while the original fibroblasts had very little cytoplasm, these later fibroblasts have a good deal. It is very reticular and vacuolated and usually stains black with Van Gieson's stain at this stage.

During the next few hours the small endothelial cells are not so noticeable; their place is taken by numbers of the larger type, which will be called macrophages for convenience. These average 6.5 to 9.5 microns in diameter, but many are extremely large and swollen and measure 24 by 10 to 12 microns. Such cells often contain whole polymorphonuclear leucocytes. The latter are now becoming more and more degenerated in appearance and are clumping to form abscesses. These abscesses, as will be shown later, persist for days. The macrophages show a very slight tendency to clump in groups of three or four, and extended search reveals a few polynucleated cells of this type, young syncytia, or giant cells. There is, however, no tendency to form what could be called a tubercle.

Up to this time the bacilli found are extracellular, they are not being phagocytosed to any extent in the first 18 hours, but lie in small masses, surrounded by a palisade of polymorphonuclears which very occasionally take up one or two bacilli that are possibly dead. The living organisms are hemmed in rather than phagocytosed.

The 14 hour lesion included a small lymph node in its sphere of influence. Although but a millimeter or so distant from the injection of carbon and bacilli, the only change noted is a marked edema of the sinuses and reticular cells and a certain activity of the germinal centers and lymphoblasts in the nodules. The marginal sinuses are distended with fluid and contain many of the smaller wandering endothelial cells, but there is no trace of carbon anywhere in the node. No tubercle bacilli can be demonstrated in its substance. The reticular cells show no particular activity, but many mitoses are to be found in the groups of endothelioid cells of the germinal centers, an observation which may be of interest to those who believe the reticulum to be the parent tissue of the macrophages or histiocytes.

The lymphocytes, which are small cells, are usually absent in practically all the sections of the series; they were found in one lesion only, that of the 9th hour. Here they were present in small numbers,

generally near blood vessels and often surrounded by a delicate network of fibrin. Were the tubercles formed from cells originating in lymphocytes, one would expect to find these cells present in quantities, which is far from being the case.

During the next 4 hours, up to the 18th hour, little change is noted; abscess formation is more marked, but otherwise the processes just outlined continue. The activity of the vascular endothelium continues up to this time and is almost exclusively limited to the smallest capillaries; the vessels which have an adventitia and well organized muscular walls do not appear to share in the process, although the adventitial cells occasionally seem to do so.

Sections made from the lesions of the third animal, representing stages from the 19th to the 28th hour, fit into the scheme which we have been constructing, in logical sequence. By 22 hours one finds that there has been a continued and marked increase in the number of large, fusiform cells resembling fibroblasts. These are surrounded by delicate basketworks of fine collagen fibrils, best seen in the Van Gieson preparations; it is also not uncommon to find the fibrils running very closely over the cells and sometimes through their cytoplasm. These cells do not, however, tend to have the tautly stretched appearance, with fanned out extremities, which is seen in the fibroblasts of rapidly growing fibrous tissue. There is a marked increase in the number of small endothelial wandering cells after the 22 hour lesion has been reached, and the increase is very noticeable up to the 30th hour, possibly indicating a second wave of migration on the part of these cells. Although the preparation representing the 30th hour was made from the first rabbit of the series of three, and the ten preceding it from the third, this section presents a picture which dovetails perfectly into the scheme, showing that the reaction does not differ materially in individuals of this species.

Tubercle bacilli are found at first in small clumps in the abscesses that form at about the 15th hour; definite phagocytosis by a number of endothelial wandering cells is first noted after 27 hours, some of these cells containing five or more bacilli without appearing to suffer materially thereby. After 21 hours the bacilli are no longer limited to smallish clumps, but are scattered broadcast throughout the abscesses.

This experiment would show, therefore, that there is at first a de-

structive action on the part of the bacilli and their toxins, in addition to the mechanical destruction caused by the injection, and that this is followed by a reaction on the part of the neighboring tissues, whereby polymorphonuclear leucocytes migrate and the endothelium of the smallest capillaries swells, proliferates, and sends out migratory cells. The polymorphonuclears surround the bacilli and form local abscesses; the mononuclears take up the carbon and later the bacilli, increase four or five diameters in size, and either wander about, or become involved in the formation of fibrils, or degenerate and die. They are actively phagocytic for the debris of the tissue and leucocytes, not only in their wandering phases, but also after they have become fusiform and apparently sessile.

The experiment also demonstrates that the cells of the tissue which has been injected with a mixture of ink and bacilli do not actively phagocytose the carbon contained in the ink, but do so only to a limited degree and then degenerate. Most of the phagocytosis is carried on by cells which wander in after 4 or 5 hours have elapsed. This bears out McJunkin's findings, but it would seem that he overstates slightly when he says that there is no phagocytosis on the part of the sessile tissue cells within 2 hours. It would appear, therefore, that one is justified in stating that, with the exception of the polymorphonuclear leucocyte and a very small percentage of original fibroblasts and their scanty progeny, practically any cell found to contain carbon particles, up to 30 hours after the intravenous injection of water-proof ink, whether intravascularly or extravascularly situated, must be considered as of probable endothelial origin. The polymorphonuclears occasionally phagocytose the carbon, but not so often as one would expect.

In the foregoing description it was shown that there were very few fibroblasts left alive after the first 2 hours following the injection of the bacilli and ink, and that cells closely resembling them began to be evident again after the migrating endothelial cells had begun to increase in size, about the 13th hour. This is in spite of the fact that extremely few mitotic figures are to be found in the surviving fibroblasts. Whence do these new cells come? The most logical supposition is that they are derived from the wandering cells of endothelial origin, particularly as they all contain large amounts of carbon and

some of them whole leucocytes or their eosinophil granules. Of course, some may be produced by the proliferation of the surviving connective tissue cells, but there is comparatively little evidence upon which to base such a conclusion. On the other hand, we can see the small endothelial cell leave the vessel, where the lining cells are swollen and increased in numbers (mitotic figures can be found in these after some searching; they were observed more frequently in the case of some guinea pigs that were experimented upon); we can see them gradually becoming filled with carbon particles and increasing in size (Fig. 1); we can demonstrate numbers of them in spindle-shaped phases, many of them in stellate or irregularly ameboid outlines; and then we find these "fibroblasts," filled with carbon and fitting perfectly into the transition picture. This transformation is stressed here, because it must be more thoroughly worked out at some future time. Of course, the cells were stained with the usual connective tissue dyes before the conclusions just indicated were reached.

The subcutaneous reactions to the tubercle bacillus were also studied from another angle, the bacilli being injected subcutaneously without any pigmented mixture. Eight rabbits were used in this experiment, the ink being given intravenously and the subcutaneous lesions produced forming a graded series, as in the first experiment. The animals were treated as follows:

Experiment 2.—Each animal received three injections of 10 cc. of 1 per cent aqueous solution of trypan blue or red intraperitoneally, at daily intervals, and then one injection every 3 days during the rest of the experiment. One loop of stock culture of bovine tubercle bacilli was suspended in 10 cc. of salt solution as in the other experiments and 1 cc. injected under the skin of the back. In two rabbits a few milligrams of carmine rubrum optimum was mixed with the suspension, but this was discontinued as superfluous in the later experiments. The injections were repeated at varying intervals, in the case of the last four rabbits once a week, and spaced down the back so as to produce five or six graded lesions between neck and tail. At first 2 to 3 cc. of Higgins' ink and distilled water, in equal parts, were injected intravenously every 3rd or 4th day, but the rabbit that survived the longest had the dose increased to 5 cc. for several doses prior to its death from epizootic pneumonia.

Most of the rabbits were killed by injecting neutral 4 per cent formaldehyde into the beating heart, under anesthesia, or were autopsied as soon after death as practicable, in which case the formaldehyde was also injected into the heart under pressure. The tissues were fixed in the same fluid and were placed in

Zenker's fluid after being sectioned in paraffin whenever this was necessary for the staining reactions. The slides were incubated for 2 or 3 hours in Zenker's fluid at 62°C. Paraffin sections were stained with Delafield's hematoxylin with or without carbolfuchsin, with Van Gieson's stain with or without carbolfuchsin, and with Mallory's connective tissue stain and his phosphotungstic acid hematoxylin. The fixation was admittedly not the proper one for the last two methods, but fair results were, nevertheless, obtained.

In the first experiment the changes that took place in the first 30 hours were observed; this one begins where the other left off and continues up to 49 days. In the first experiment there was little or no evidence of true tubercle formation, at best a slight tendency to clumping was exhibited by the wandering cells. In the series now under discussion the reaction was found to be invariably the same, the formation of an abscess with small tubercles scattered around its periphery. These are engulfed in the advancing caseous abscess and new ones form at the constantly enlarging border. For this reason and with this heavy dosage it was impossible to obtain anything resembling the graded series of the first experiment, or that in which agar was used as an irritant. On the 5th day the small peripheral tubercles begin to form and spread into the outlying tissue; any lesion older than this merely shows the same condition on a wider scale. Thus we are limited to the study of these small tubercles, which are typical and range from submiliary to confluent in nature. There is no appreciable difference to be noted in the lesions from one or another animal of the series, except for the variation in the amount of carbon and vital dye contained, which is readily explained by the fact that some of the animals were killed soon after, others a day or more after the last injection of ink or dye. Repeated injections of tubercle bacilli seemed to cause no variation in the type of lesion produced; the later ones differed in no respect from the earlier ones. In two of the rabbits neighboring lymph nodes became infected through the lymphatics, and tiny tubercles, without much caseation, were produced in them.

The true tubercles, best studied after 2 weeks of development, on account of their greater numbers, consist of large mononuclear cells, liberally sprinkled with carbon particles, and of lymphocytes, the latter always free from this pigment. The endothelium of the ves-

sels in the invaded tissue always shows plentiful carbon, in some cells sparsely scattered, in others so liberally distributed as to obscure the cell anatomy. Sometimes the mononuclear cells are found free in the lumina of these capillaries, containing varying amounts of carbon, but they are seldom found in the older preparations, in the circulating blood of the larger vessels. So striking is the distribution of carbon in the tubercles that one can glance over a slide with a low power objective and immediately locate the lesions by reason of their dusky color. In one of the animals in which trypan red was used, the tubercles were not only dusted with carbon but were also stained brick-red.

In giving a detailed account of the relation of the coloring matter to the cells it will be necessary to take up each substance in turn. The carbon is found almost invariably in mononuclear cells; little is taken up by the polymorphonuclears, although no citrate was combined with the injection. These cells are found (*a*) free in the circulating blood near or in the lesions, (*b*) in the endothelium of the blood capillaries, seldom in that of the lymphatics, (*c*) in the epithelioid cells forming the tubercles, where the carbon is usually in a fine, more or less separated form, although it may be found very liberally distributed as shown in Fig. 2, and (*d*) in the syncytia, or giant cells, formed by the fusion of these cells, as would be expected from what has already been described in the experiments with agar injections.

It would have been very difficult to trace the carbon from the vessels to the tubercles with only this experiment as a guide; fortunately the first experiment described above makes it clear that this substance is taken up by the cells composing the smallest capillaries and that these cells then migrate and congregate to form the tubercles. The agar experiment, too, makes the mechanism clear. It is probable that the cells of the capillary walls swell and proliferate in response to the action of the bacilli in the neighboring tissue and become more permeable to the carbon, or more sticky, attracting it and then taking it up. Although it was not possible to find many mitotic figures in the rabbit series, they were found with comparative ease in the endothelium of the smaller capillaries in infected guinea pigs which were studied during preliminary experiments, and were easily found in the lungs of rabbits experimented on in a subsequent series. Carbon is also present in cells containing fibrillæ which stain with acid fuch-

sin in the Van Gieson stain and Mallory's connective tissue stain. A few of them show blue fibers with the latter stain.

The trypan dyes are found in the usual sites, mononuclear phagocytes, or macrophages, and connective tissue cells. Trypan red is also occasionally taken up by the polymorphonuclears in lesions of the subcutaneous tissue and lymph nodes, where there is evidence of stagnation in the circulation. It is sparingly found in the capillary walls under like circumstances. Unlike trypan blue it stains collagen fibers diffusely, and like that stain colors necrotic tissue in the same manner. As the mononuclear cells containing it increase in size and take up more bacilli, the dye granules tend to disappear, the stain reappearing diffusely once necrosis has set in. The acid used in differentiating the carbolfuchsin stain (20 per cent nitric) changes the trypan red to a dirty brown and tends to diffuse it throughout the cell, but the granules can still be made out as brownish masses, while trypan blue is diffused, but unaltered in color.

A 1 per cent solution of trypan red turns the same color *in vitro* if 20 per cent nitric acid is added; the original color is restored by neutralizing with ammonia. Trypan blue is only slightly affected by the acid, becoming a little more reddish; the blue is more or less completely restored by neutralization.

In the cases in which powdered carmine is injected with the bacilli, it is found in the phagocytic cells and fills them more or less completely, according to their size and proximity to the pigment; the smaller endothelial wanderers contain a few granules, the larger forms may be quite filled with them. Tubercle bacilli are found in these cells, and one may find them containing granules of benzidine dye, bits of carmine, carbon particles, and bacilli at the same time.

In this experiment, then, the findings of the first are confirmed in that all these substances are phagocytosed by the same cell in the infected areas and that this cell forms the tubercle and is, therefore, the epithelioid cell, and that it originates in the walls of the smallest capillaries and is, for this reason, endothelial. The more one follows out this line of investigation, the more impressed does one become with the fact that the endothelium of vessels whose walls are made up of several layers of cells and fibers seems to be more specialized and, perhaps for this reason, less affected by outside influences than the cells which compose the capillary walls.

That the lymphocytes play a part in the formation of the tubercles is evident, but they appear relatively late, and it is doubtful that this rôle is a formative one; we are thrown back upon hypothesis, as far as they are concerned. There is no evidence from these experiments that they are transformed into the much larger and paler tuberculous epithelioid cell; they do not contain any of the colored substances used and it is difficult to find any intermediate stages that would fit them into such a transition picture. Neither has the lymphoid tissue proper any affinity for the benzidine dyes or carbon; it is only the reticular cells that take them up. This is true of both lymph nodes and spleen.

In this, as in the first experiment, phagocytic cells are found which stain with the ink and also contain bacilli, while they are intimately connected with fibrils which take acid fuchsin intensely (Figs. 6 and 7). True collagen fibrils can be demonstrated in some of them. Both in the case of the reticulum of the tubercle and the collagen fibers which surround the lesion, too dense an accumulation of delicate fibrillæ is found to warrant our considering all of them preexistent; they seem to be newly formed. This is particularly evident in connection with the tubercles in the lymph nodes, where the fibrous reticulum is normally very scanty and delicate, and the fibers which we find seem to be too intimately connected with the carbon-bearing cells not to be in some way related to them. These fibers stain darkly with phosphotungstic acid hematoxylin, blue or brownish blue. The further discussion of these fibrils must be relegated to another paper, for the principles involved are too far reaching to be taken up without thorough investigation.

The lesions in the lymph nodes are, in every particular, similar to those studied in the subcutaneous tissue and the connective tissue of the muscles infected. They are invariably perivascular, and the small blood vessels in or near them show the same proliferative changes in their walls. The cells forming these vessels are indistinguishable from those composing the tubercles; in fact the lumen of the vessel becomes a canal in a mass of tissue composed of homologous cells, most of which show carbon in varying amounts. Syncytia are abundant and usually show many carbon granules. In the normal portions of the nodes little carbon is to be found, save in cells circu-

lating in the blood capillaries; the reticular cells do not ordinarily take up much of it unless included in the lesions.

Comparing the findings of these experiments with those of the first one, in which agar was the exciting agent, we find that the reaction is essentially the same in its inceptive stages, but that it differs from the latter in that it is more than mechanically destructive, that it is progressive, and that the action of the exciting agent (tubercle bacilli) continuing over an indefinite period of time repeatedly frustrates the reparative process. In this way, instead of a mass of simple syncytia and subsequent organization, there is only an attempt at their formation, which is then terminated by the destructive activity of the bacilli, with resulting caseation. Therefore, whether the bacilli are intravascularly or extravascularly situated, tuberculosis is primarily a disease the lesion of which is formed by cells originating in the smallest branches of the vascular system; it will be shown in the next paper (Foot, 1920) that this is also true in the case of experimental pulmonary lesions.

General Discussion of the Literature on the Subject.

The origin of the epithelioid cell has been, as already stated, a constant subject of dispute; the standard text-books of pathology, published in recent years, show that there is still no unanimity in regard to this point. MacCallum gives a very good review of the various theories, in conclusion showing that he is definitely inclined to favor that of Maximow (1906, 1909, 1910), which traces the epithelioid cell to the lymphocyte. Adami and Nichols, and Mallory are in favor of their endothelial origin. Askanazy and Lubarsch, writing for Aschoff's system, take the more conservative stand that the connective tissue, vascular endothelium and epithelium, as well as the mesothelium in some instances, are all responsible for these cells. Delafield and Prudden believe that they have their source in the endothelial and connective tissue cells. One might continue to quote opinions quite as divergent.

The literature on experimental tuberculosis dates back to the early seventies and is rather voluminous; most of the important authorities will be found listed in the references at the end of this paper; for a more complete bibliography the reader is referred to Kockel's article. In the early history of the study of the origin and histogenesis of the tubercle two opposing groups are recognizable, the adherents of Baumgarten (see Kockel), who claimed the cells of the fixed tissues (including the vascular endothelium) as the parents of the epithelioid cell, and the followers of Metschnikoff, who saw in the process an exudative phe-

nomenon, or migration of the mononuclears from the blood stream, with subsequent proliferation of these cells in their new situation. Curiously enough, the earliest observers in 1871 and 1873, were inclined to consider the vascular endothelium alone responsible for these cells; with time technique and theories became more and more elaborate and this view-point was lost, or rejected, by most authorities. Of late it has tended to come more and more into prominence.

The weight of opinion seems to favor the endothelium and fibrous connective tissue as the origin of the epithelioid cell, with the epithelium playing a similar part in the case of the eye, lung, and kidney. That the endothelial cell is its parent in the case of the liver is claimed and, it would seem, proved, by Kockel, Miller, Oppenheimer, Goldmann, and Evans, Bowman, and Winternitz. Watanabe and Sewell are of the opinion that the alveolar epithelium of the lung may be the main source of the epithelioid cells of experimental pulmonary tubercles, while Wechsberg believes the endothelium to be their origin. We shall have occasion to take this up in detail in the next paper, so the subject will not be discussed further here. Kostenitsch and Wolkow, who have worked on the eye, think that both the endothelium and the pigmented epithelium share in the process. The kidney has furnished enough evidence of epithelial proliferation for the same authors to allot a share in the formation of renal tubercles to the tubular epithelium. As to tubercles produced elsewhere in the body, one finds little theory based on experimental evidence.

Theories as to the formation of tuberculous syncytia, or giant cells of the Langerhans type, appear in almost every paper; Lambert's work on the foreign body syncytium *in vitro,* and Forbes' and my own, in connection with its production in response to injections of sterile agar, are probably applicable to this cell in the tubercle. There seems to be nothing particularly specific to tuberculosis in its presence, when its appearance in the gumma and other forms of chronic inflammation are considered.

The most interesting confirmation of the findings of the two sets of experiments described in this paper is to be found in the articles of Miller and Kockel. The former shows that the first reaction to the tubercle bacilli in the liver is an exudate of polymorphonuclear leucocytes, which surround the organisms and arrange themselves in "little heaps." He finds the bacilli phagocytosed for the first time 100 hours after injection, considerably later than when noted in the present experiments. He puts the first appearance of lymphocytes at 6 to 9 days, which corresponds accurately with what has been found in the case of pulmonary lesions (Foot, 1920) so that he, too, would seem to rule these cells out of the earlier reaction.

Kockel experimented with injections of farina into the portal vein and produced pseudotubercles, the formation of which was analogous to that of true tubercles, except that whereas the pseudotubercles became vascularized in from 3 to 4 days, the true tubercles showed no inclination to undergo a similar change. The smaller type of endothelial cell was much more in evidence in the pseudo-

tubercle than in the true tubercle, as is the case if reactions to agar are compared with those to tubercle bacilli. Necrosis was present in the tubercles formed in response to the farina; Kockel argues that this is due to stoppage of vessels, which he found to be thrombosed in many cases, rather than to the action of toxins, as one would suspect in the case of true tubercles. Vissman, however, finds that dead tubercle bacilli, when used in such an experiment, while they produce tubercles, cause no caseation thereof, a fact not in accord with Kockel's theory. Morse reported and demonstrated that tubercles, apparently in every way similar to those produced in the rabbit lung by injection of bacilli, could be formed in response to the injection of waxes extracted from dead cultures of the bacilli. Not only were these tubercles cellular structures, but caseation was also present and widespread. This is at variance with the findings of Vissman. The explanation given by Morse is that the leucocytes are hindered in their phagocytic activity by the presence of the wax.

Kockel states in this connection:

> From the peculiar behavior of the endothelium in the periportal granulation tissue and in the young intracapillary tubercles it may be assumed that caseation is less likely the result of direct action of tubercle bacilli on the cells, than of the inhibition of the formation of new vessels and the obliteration of preformed vessels owing to the extreme proliferation of their endothelium which is the result of this injurious action.

He argues that the endothelium proliferates in tuberculosis, as it would in any granulation tissue, for the purpose of forming new vessels; but, owing to a stoppage in the flow of blood and the *vis a tergo* thereof, this proliferation takes the form of sheets or plugs of cells, which are then known as tubercles. That this theory is largely true is well shown in some of the slides which have been studied in the present work, particularly in the case of the lymph node infections in the second series of experiments; that the absence of the *vis a tergo* of the circulating blood plays as important a part as he would ascribe to it cannot be so readily accepted.

As far as the theories of the different schools are concerned, we have already seen how much evidence there is in favor of the endothelial origin of the epithelioid cell. That Metschnikoff's theory as to the exudate of mononuclears from the blood stream, with subsequent multiplication *in situ*, is at least partially correct is evident when one considers that the carbon-bearing endothelial cells are found in the lumina of vessels in the infected areas. That they come from very distant parts of the body seems unlikely in view of the fact that there is such marked evidence of local proliferation of the endothelium; that they are free in the circulation remains undisputed; and that they may migrate and, in the tubercles, join other endothelial cells,

which left the vessels by the external route, must be admitted. Mitotic figures are easily found in the smaller endothelial cells after they reach the site of inflammation, which would further corroborate Metschnikoff's view, although it cannot be determined whether or not these cells were ever free in the circulation. It seems probable that Metschnikoff's theory is correct, if taken with reservations; such cells as he refers to probably migrate in numbers much smaller than those of the vascular endothelial cells which leave the walls by the external route, and they probably come from the walls of capillaries in the proximity of the lesion, rather than from so distant a point as, for instance, the spleen or bone marrow. There is little to indicate that they come from the lymph nodes, even in the immediate vicinity of the lesion.

That the fibroblast is the parent of the epithelioid cell is not borne out by the evidence which is at hand, although the reverse may be true of the fibroblasts which tend to wall off the lesion, as has been indicated. As to the lymphocyte, it has already been stated that nothing has been found to show that it develops into the larger cell; it is present in the tubercle and therefore is a component thereof, just as is its derivative the plasma cell; but it appears too late to be considered as a potential epithelioid cell. This paper does not deal with the epithelium or the mesothelium; they will be considered as possible factors in later studies. Part of the work has already been completed and it may be said in advance that the epithelial cell may be ruled out in the case of experimental pulmonary tuberculosis as a potential epithelioid cell.

SUMMARY.

1. The epithelioid cell is of definitely endothelial origin.

2. The only reliable means of identifying and tracing this cell is, at the present time, a colloidal suspension of carbon, injected intravenously. Benzidine dyes will not accomplish this if used alone.

3. There is little evidence that the local tissue elements take an active part in the process of tubercle formation, until after the lesion is formed; the reaction is, in a sense, exudative, since the lesion is produced from cells which migrate to the site of inflammation.

4. The lymphocyte appears late and is not to be considered as a potential epithelioid cell; its presence in the tubercles is as yet unexplained.

BIBLIOGRAPHY.

Adami, G. J., and Nichols, A. G., The principles of pathology, Philadelphia and New York, 2nd edition, 1911.

Askanazy, M., in Aschoff, L., Pathologische Anatomie, Jena, 2nd edition, 1911, i, 165.

Delafield, F., and Prudden, T. M., Handbook of pathological anatomy and histology, New York, 6th edition, 1901.

Evans, H. M., Bowman, F. B., and Winternitz, M. C., *J. Exp. Med.*, 1914, xix, 283.

Foot, N. C., *J. Med. Research*, 1919, xl, 352.

Foot, N. C., *J. Exp. Med.*, 1920, xxxii, 533.

Forbes, A., *J. Med. Research*, 1909, xx, 45.

Goldmann, E. E., *Beitr. klin. Chir.*, 1912, lxxviii, 1.

Kockel, R., *Virchows Arch. path. Anat.*, 1896, cxliii, 574.

Kostenitsch, J., and Wolkow, M., *Arch. méd. exp. et anat. path.*, 1892, iv, 741.

Lambert, R. A., *J. Exp. Med.*, 1912, xv, 510.

Lubarsch, O., in Aschoff, L., Pathologische Anatomie, Jena, 2nd edition, 1911, i, 534.

McJunkin, F. A., *Arch. Int. Med.*, 1918, xxi, 59.

McJunkin, F. A., *Am. J. Anat.*, 1919, xxv, 27.

McJunkin, F. A., and Charlton, A., *Arch. Int. Med.*, 1918, xxii, 157.

MacCallum, W. G., A textbook of pathology, Philadelphia and London, 1916.

Mallory, F. B., The principles of pathologic histology, Philadelphia and London, 1914.

Maximow, A., *Arch. mikr. Anat.*, 1906, lxvii, 680.

Maximow, A., *Arch. mikr. Anat.*, 1909, lxxiii, 444.

Maximow, A., *Arch. mikr. Anat.*, 1910, lxxvii, 1.

Miller, J., *Beitr. path. Anat. u. allg. Path.*, 1902, xxxi, 347.

Morse, P., Paper read before the 20th annual meeting of the Association of American Pathologists and Bacteriologists, New York, N. Y., April, 1920.

Oppenheimer, R., *Virchows Arch. path. Anat.*, 1908, cxciv, suppl., 225.

Sewell, W. T., *J. Path. and Bact.*, 1918–19, xxii, 40.

Vissman, W., *Virchows Arch. path. Anat.*, 1892, cxxix, 163, quoted by Miller.

Watanabe, K., *Beitr. path. Anat. u. allg. Path.*, 1902, xxxi, 367.

Wechsberg, F., *Beitr. path. Anat. u. allg. Path.*, 1901, xxix, 203.

EXPLANATION OF PLATES.

Plate 84.

Fig. 1. Semischematic camera lucida drawing of carbon-containing endothelial cells from the exudate, to show their enlargement over a period of 24 hours. Cells *a* to *d* are the earliest type seen; *e* to *g* can be found after about 8 hours following the injection of bacilli and lampblack suspension. Cell *h* is an extremely large form, seen in the latter part of the series of slides, at about 24 or more hours after injection. × 1,120.

Fig. 2. A tubercle found in the second experiment, with an excessive amount of carbon particles. 42 days after injection. Camera lucida drawing. × 1,120.

Plate 85.

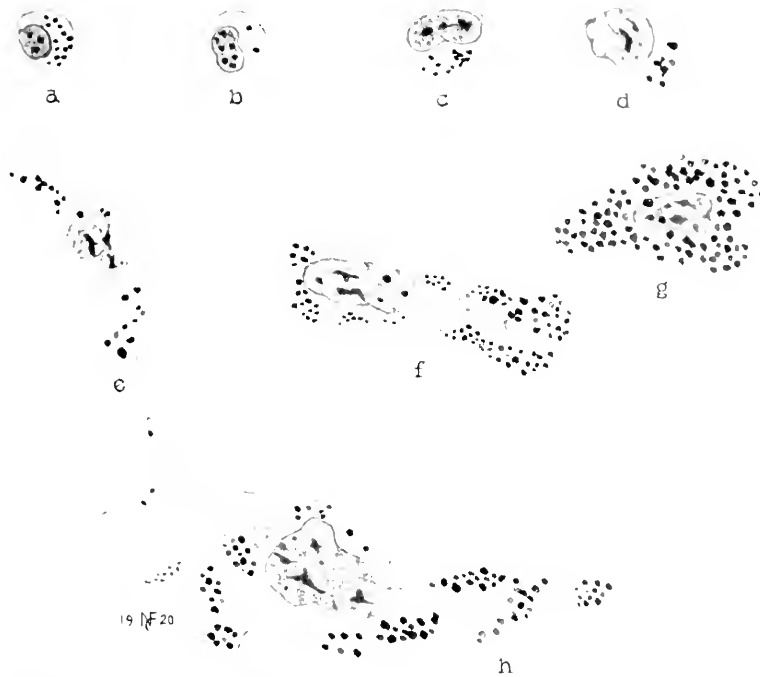
Fig. 3. Photomicrograph of endothelial leucocytes in early exudate. × about 1,000.

Fig. 4. Similar cells in denser tissue. × 1,000.

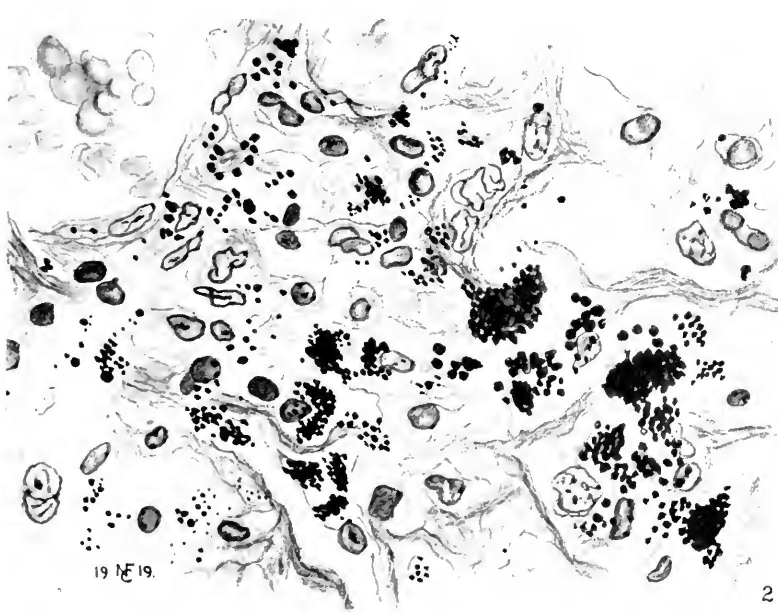
Fig. 5. Two fusiform types, one burrowing into the dense tissue, with carbon particles in its curved "tail." × about 1,000.

Plate 86.

Figs. 6 and 7. From tubercles on the 48th day after injection. Note the distribution of carbon in both, of the reticulum in Fig. 6, and of the bacilli in Fig. 7. × about 800.



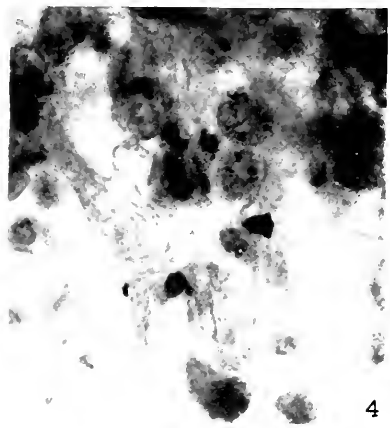
1



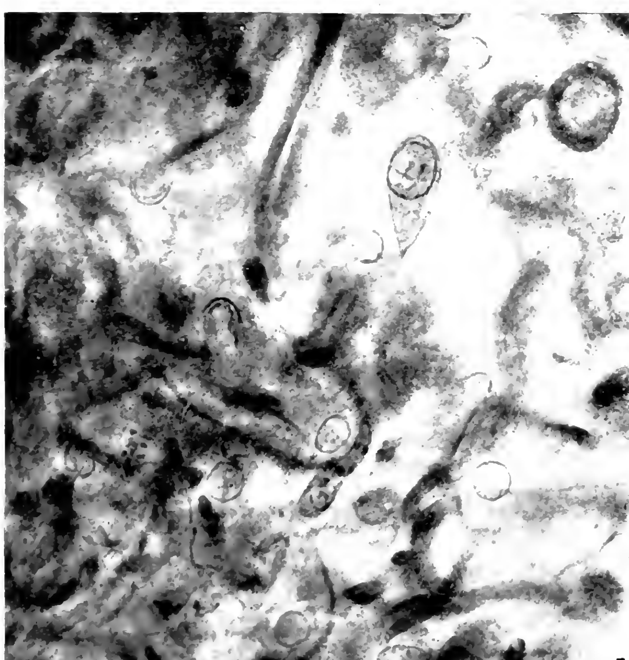
2

(Foot: Endothelial reactions. II.)

3

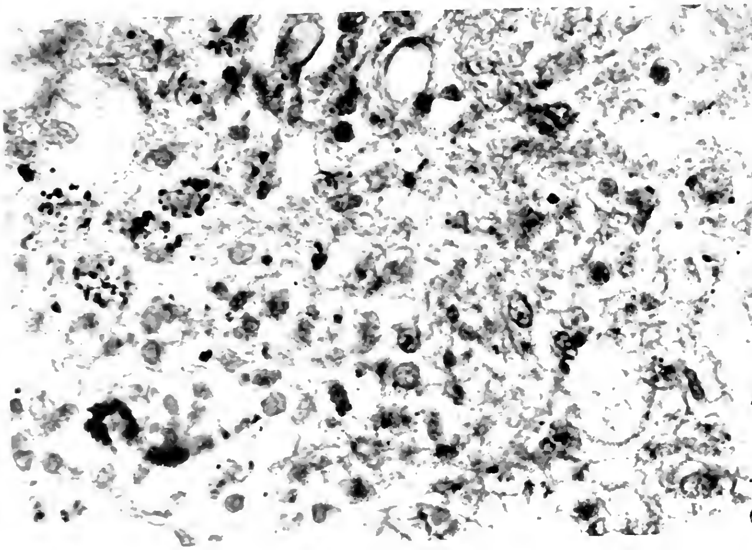


4

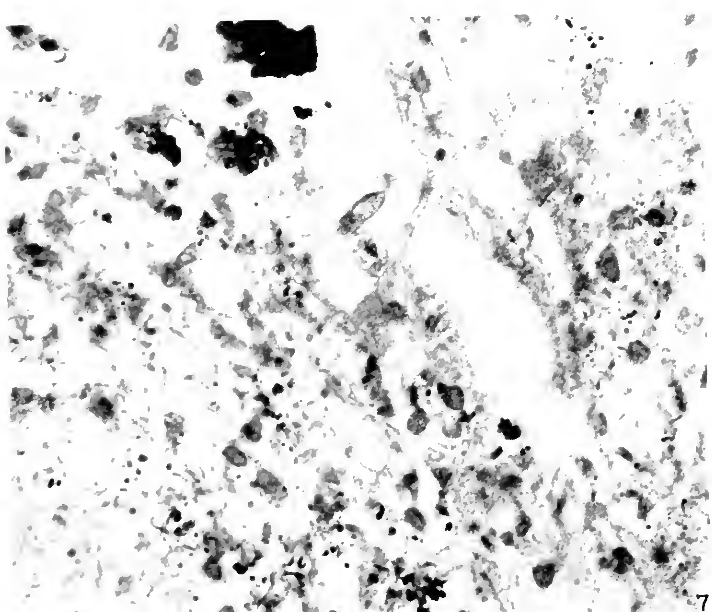


5

(Foot: Endothelial reactions. II.)



6



7

(Foot: Endothelial reactions. II.)

STUDIES ON ENDOTHELIAL REACTIONS.

III. The Endothelium in Experimental Pulmonary Tuberculosis.*

By NATHAN CHANDLER FOOT, M.D.

(*From the Department of Comparative Pathology, George Fabyan Foundation, Harvard Medical School, Boston.*)

Plates 87 and 88.

(Received for publication, May 26, 1920.)

In two preceding papers of this series (Foot, 1919, 1920) the reaction of the endothelium to a foreign body, agar, was discussed, as well as its reaction to the tubercle bacillus when introduced subcutaneously. It is the purpose of this communication to consider the reaction to this organism when injected through the trachea into the lungs, with special reference to the origin of the epithelioid cell of the pulmonary tubercles. It has been shown in the second article of this series (Foot, 1920) that it is the endothelial cell which forms this component of the subcutaneous tubercle. Wechsberg, writing in 1901, apparently considers the endothelium to be the source of these cells in the pulmonary tubercle, but both Watanabe and Sewell have taken the stand that the alveolar epithelium plays an important part in their formation. It is in an attempt to answer this question conclusively that the present experiment has been undertaken. A general discussion of the theories on the subject will be found in the second paper (Foot, 1920) and will, therefore, be omitted here. There is strong evidence that the endothelial cell is called out in response to foreign bodies and to the presence of bacteria of a low grade of virulence. That this is true in the case of the liver has already been shown. There does not seem to be any reason why there should be a different response in any other part of the body.

Wechsberg injected tubercle bacilli into the lung by way of the trachea and studied the reactions in sections stained mostly with a view to getting an idea of the damage done to connective tissue. He found that the elastic fibers were destroyed within 6 hours after the injection and that there were defects in the endothelium of the capillaries, through which polymorphonuclears and endothelial cells migrated into the alveoli. He summed up his findings thus: (1)

* An abstract of this article was presented at the 20th annual meeting of the Association of American Pathologists and Bacteriologists, New York, N. Y., April 3, 1920.

There is first a destruction of fixed tissue cells. (2) New formed or migratory cells are injured only in as far as the formation of new vessels is hindered and connective tissue growth is hampered and incomplete. Giant cells are formed. (3) There is a second phase of destruction with caseation. In view of his evidence as to the endothelial origin of the tubercles, it is unfortunate that he does not commit himself more definitely on this head.

Watanabe used the same methods and killed the animals 12, 24, 48, and 96 hours after injection. He found bacilli in "swollen epithelial cells" of the alveolar walls, in the epithelium of the bronchi, and in cells which might be endothelial in origin—"mononuclear leucocytes." He found nothing to indicate that the fibroblast took them up.

Sewell was the first to use vital stains, in the modern sense, in connection with experiments on pulmonary tuberculosis. In his paper he quotes an experiment done by Slavjansky in 1869. This investigator injected suspensions of indigo into the trachea of rabbits and after 2 or 3 days introduced 5 to 7 cc. of a fairly thick "solution" of cinnabar into the jugular vein. 2 days later he killed the animals and found some cells lying free in the alveoli and containing indigo, others containing cinnabar, and a third group free from either. The alveolar epithelium contained indigo and was otherwise unchanged. If, however, he injected the cinnabar into the blood immediately after introducing the indigo into the trachea, he could find cells in the alveoli and bronchial mucosa containing granules of both pigments. He concluded that, in the first case, the cells containing the cinnabar had wandered out of the vessels (thus indicating the possibility of their being of endothelial origin) and finding no indigo free, remained unaffected thereby; while in the second case, they arrived before the supply of indigo had been exhausted and therefore contained both kinds of pigment.

Tchistovitch repeated these experiments in 1889, substituting bacteria for the indigo in the tracheal injection and using carmine intravenously. The animals were killed in 24 hours. Carmine-laden cells were found in the alveoli, and there was also phagocytosis of the bacteria by small cells which contained no carmine granules; he could find no cells containing both, which is not consistent with my findings, as will be seen later.

Sewell injected a suspension of bovine tubercle bacilli in lithium-carmine into the trachea, using 2 cc. at a time. The rabbits were vitally stained with trypan blue and were killed 24 and 72 hours after introducing the bacilli. In one animal he found cells stained with the blue dye lying in the alveoli. As this rabbit showed an extensive pneumonia and pleurisy, he concluded that these cells migrated in response to the infection at a time prior to the injection of the carmine and bacilli, but after the trypan blue staining was complete. Some of these cells contained trypan blue granules, carmine, and tubercle bacilli. The other animal, however, showed only carmine in the free cells. He injected a fine suspension of India ink into the trachea of rabbits vitally stained with lithium-carmine and killed them 3, 6, 15, and 24 hours later. Cells stained with car-

mine did not appear until after 24 hours, but cells containing carbon were found before this and appeared to originate from the epithelium near the alveolar angles. Carmine injected into the trachea gave excellent staining reactions in the alveolar epithelium after 3 hours and trypan blue after 6 hours. He injected 10 cc. of a mechanical suspension of India ink into the ear vein of a rabbit and found none whatever in the lungs.

This investigator did a number of experiments with various dyes injected alone into the trachea of rabbits and guinea pigs, which give an idea of the lesions produced by the injection of such dyes, unmixed with bacteria, while they also serve as valuable control experiments. Quoting Briscoe as to the three possible sources of the mononuclear cells found in the alveoli in pulmonary inflammation, he says that these may be (1) the macrophages in the interstitial tissue near the alveolus, (2) the alveolar epithelium, and (3) specialized epithelium not concerned in respiratory function. His frequent allusions to the similarity of the epithelial and endothelial phagocytes and the fact that he admits that the only means of ruling out the latter in these experiments is the use of intravenous dyes which do not immediately appear in these cells, but require the lapse of a day or more to make their presence known, all indicate the difficulty that is encountered in drawing conclusions from such experiments. Moreover, the carmine that he used intravenously is not specific for the vascular endothelium *in situ* and the mechanical suspension of India ink, upon which he based his final conclusions, did not show in the lungs at all; whereas, had he used a colloidal suspension of carbon, which never fails to show, his conclusions might have been different. In summing up he says: (1) The alveolar cells are endowed with the full phagocytic powers of the histiocyte. (2) Their behavior under the action of a stimulus is similar. (3) When certain dyestuffs are offered to them in colloidal solution, they store up granules of the dye in their protoplasm in the same manner as the histiocytes. He clearly indicates his belief that, in the case of experimental pulmonary tuberculosis, the epithelioid cells are really epithelial cells which are the first to respond to the stimulus of the infection.

Method.

In the experiments which are the basis of this paper I have used two pigments, lithium-carmine and Higgins' water-proof ink, which is a colloidal suspension of carbon in water, with an emulsifying agent and a little camphor. The former was employed solely for intratracheal injections, the latter for the intravenous; both of them are colloidal suspensions. The carmine suspension is prepared by mixing 4 gm. of Grübler's carmine rubrum optimum in 100 cc. of a saturated solution of lithium carbonate, boiling, and filtering through filter paper. The ink suspension is a mixture of equal parts of commercial

Higgins' water-proof ink and distilled water. The carmine suspension is sterilized before use; the ink mixture need not be sterilized if sterile distilled water is used, as the ink contains enough camphor to keep it free from bacterial growth.

The fundamental principles involved are the supplying of two substances of different color, one through the air passages, the other through the circulation; that used in the latter instance must be one that will be taken up by the endothelial cells *in situ* and I know of only one such, a colloidal suspension of carbon, either in the ready made form just stated, or in that recommended by McJunkin. The latter is a 4 per cent suspension of finely triturated lampblack in normal salt solution, with 1 per cent of gelatin added to it. In this way the alveolar epithelium will be offered a red dye, the endothelium of the capillaries a black one, the origin of the cells that respond to the stimulus of the tubercle bacilli being readily traced by their color. The question of the specificity of the carbon for the vascular endothelium has been presented at length in the two preceding papers of this series, so any further discussion will be omitted here.

Histology of the Alveolar Epithelium.

Before proceeding to the consideration of the experiments done along these lines, it will be necessary to discuss the histology of the alveolar epithelium, for much error may result from misconceptions as to its structure. Once a definition of a typical alveolar epithelial cell has been accepted, the investigator will know what to look for when searching for such cells and will realize that not all are globular, with the bean-shaped nucleus and other characteristics of the endothelial leucocyte.

The epithelium of the alveoli, as well as that of the infundibula, is not a continuous layer of nucleated cells, but a membrane of varying thickness, composed of several types of cells, non-nucleated plates, and flanges, or processes, of nucleated cells. There are areas where one finds groups of nucleated cells very similar to those of the endothelium and practically indistinguishable from them. There are more or less cuboidal nucleated cells which extend almost through the alveolar wall and around which the capillaries twist, but whose alveolar

surface is more or less flared out to become continuous with adjacent cells. Thirdly, there are large, flat cells with broad plates extending from them, in which the nucleus is situated in a small mass of protoplasm more dense than the rest. These extending plates may represent similar cells that have lost their nuclei, or the processes, or flanges, of the flat, nucleated cells; both theories are advanced, the former being the most commonly encountered.

A cross-section of one of these plates is extremely difficult to see, as the cytoplasm has a refractive index that approaches that of Canada balsam very closely; but they can be made out under suitable conditions. It is possible to demonstrate them by injecting the fresh lung with a 0.25 per cent solution of silver nitrate, when the demarcation becomes evident. In my preparations they are best seen in tangential planes of section in material fixed in neutral formaldehyde; Helly's fluid shrinks them and causes fenestration of the cytoplasm. How they are formed from the cuboidal epithelium which precedes them in the fetus is still a matter of dispute. Bremer's work on the opossum lung would indicate that they are, in reality, flanges, or processes, of epithelial cells that are stretched out thin over capillaries. The reader is referred to such text-books as those by Ellenberger, Minot, Stöhr, etc. That most of these cells do not resemble the endothelial leucocyte can be seen at a glance; that they desquamate in the forms just described is readily demonstrable.

The group of roughly cuboidal cells is the most difficult to classify in regard to origin. Without ink injections the question seems unanswerable, as they look exactly alike; with ink in the cytoplasm of some of them, we can assume that these are endothelial. It would seem advisable to study the question of cell types in animals that are vitally stained, using a tridimensional wax reconstruction technique. That some of the cells in the groups which so closely resemble the endothelium may really be such, must be kept in mind. They might migrate from the capillary walls and collect in these situations, just as they do in the other tissues in the body, there to act as potential phagocytes in an organ where phagocytosis is very necessary. This is not a new idea; that many endothelial phagocytes are normally present in the lung is well recognized; their origin in the capillary walls and storage in the clumps of cells already described would, it seems, be perfectly natural.

Reaction to Pigments Alone.

Before describing the experiments in which tubercle bacilli were injected into the trachea, it would be well to know the reaction to pigments alone. In one rabbit ink was injected intravenously, and the animal died about an hour later while going under ether. Two rabbits were injected intravenously with 5 cc. of Higgins' ink and distilled water in equal parts and intratracheally, under an anesthetic, with 2 cc. of the lithium-carmine preparation. One was killed 24 hours later, the other 6 days later. The lungs and bronchial tree were removed *en bloc* and the former distended to three-quarters their normal expanded volume by injecting 4 per cent neutral formaldehyde. They were then sliced thin and fixed either in formaldehyde or Helly's fluid. They were stained with Delafield's hematoxylin, eosin-methylene blue, and Mallory's phosphotungstic acid hematoxylin.

In the animal in which the ink was used alone, the carbon is distributed in the endothelium of the interalveolar capillaries, and to a slight degree in the circulating mononuclear leucocytes, while some of it lies free in the circulating blood. It is also found in large cells in the alveoli, as described below. In the two other animals the ink distribution is the same, more appearing in the large cells in the alveolar spaces after 6 days than after 24 hours. There is a slight polymorphonuclear leucocytosis, with a scanty exudate of these cells after 24 hours; this is not seen in the 6 day preparations.

The large intraalveolar mononuclears resemble macrophages in every way; they are of irregularly globose, or obtusely angulated outline and have vesicular reniform nuclei and may contain vacuoles. They lie in the lymph spaces just below the epithelium, or free in the alveolar sac, or fitted into indentations in the alveolar surface of the epithelium, which surrounds them on three sides. Sometimes they seem to be interposed between two epithelial plates, as though penetrating the lining, and they can be seen, not infrequently, leaving the capillaries and lying athwart the lymphatics, with a portion of their cytoplasm penetrating the epithelium, in the act of migration. These cells are all important in the work at hand, for they play the principal part in the reaction to the tubercle bacillus in the air-borne infection and are evidently the cells which Sewell thought to be epithelial.

They show a few carbon granules as early as 1 hour after injection and take it up steadily for 6 or 7 days, when the experiments terminated. A few may be loaded with the ink particles, especially when in the alveolar wall; sometimes they are filled with carbon when free in the air space; usually, however, those that migrate contain less than those that do not. The foreign matter may hinder their motility to some extent. A little carbon is found in occasional polymorphonuclear leucocytes, but as a rule it is confined to the mononuclear cells. It is never found in the bronchial epithelium, or in any cells of clearly epithelial type in the alveoli. That it could be taken up by the epithelium from the blood stream seems highly improbable in the light of our present knowledge of the selectivity of the endothelium for this substance. The ink never seems to lie free in a position where the epithelial cells could phagocytose it, and the idea that it could be passed from the endothelial to the epithelial cells is not worth serious consideration.

The mononuclear migrating cells take up the carmine diffusely at first; after 5 hours, as seen in later series of sections, only one or two granules appear; after 24 hours the granular stain is complete and very beautiful. In the 6 day control animal these cells are found scattered in twos and threes in the alveolar sacs and bronchial lumina, or grouped in small masses in the alveoli and infundibula. They do not tend to form syncytia and show an intense granular carmine stain. One can also find them in the lymphatics and lymph nodes of the lung tissue and mediastinum. The epithelium of the bronchi and alveoli does not take the carmine except in a very faint, irregular, and diffuse manner. Thus the tissue reaction to the pigments alone is of a very slight and transitory character.

Experimental Pulmonary Tuberculosis.

In the actual experiment on the reaction to the tubercle bacilli the following procedure was carried out. Two stock strains of bovine tubercle bacilli were utilized and two methods were employed in preparing the suspensions used. In the first case one or two loops of dry culture on glycerol agar were rubbed up as finely as possible with a flat platinum needle and gradually suspended in 10 cc. of the lithium-carmine preparation used in the control tests, to be mechanically shaken for from ½ to 1 hour. The bacilli were first rubbed into a paste, more fluid was added, and the needle was bent into a J and rapidly spun between the

thumb and index finger until a fairly uniform cream was obtained. In the second instance a glass bulb pestle was used to triturate the same amount of culture into a thin film on the walls of a test-tube, fluid being added gradually and the whole shaken as before.

A series of rabbits was then injected intravenously with dilute Higgins' ink as in the controls; they were then anesthetized and 2 cc. of the suspended tubercle bacilli in lithium-carmine introduced into the trachea, just below the larynx, by means of a hypodermic syringe and needle. After a lapse of 1 hour, 5 hours, 1, 2, 3, 4, 5, 6, and 7 days the rabbits were killed by anesthetizing and injecting neutral 4 per cent formaldehyde into the beating heart. The lungs were then removed and treated as in the controls. The 5 and 24 hour lesions were produced in duplicate, one pair of rabbits receiving one strain, the other the second strain of tubercle bacilli.

The lesions resulting in all cases varied slightly with the individual and with the type of suspension used, the coarser type causing changes more like the usual tubercles and tuberculous bronchopneumonias, while the finer caused lesions that resembled caseous lobar pneumonia and were accompanied by fibrinous pleuritis (*cf.* Sewell's case).

The development of the experimental lesions, observed at the intervals indicated, proceeds as follows: First there is a widespread desquamation of the epithelium of the affected alveoli, often with destruction of their entire wall, including the capillary; large, flat cells, plates, and the debris thereof are found lying free in the air sacs and the lumina of the bronchi. Sometimes there is swelling of the epithelial plates, which peel off at their edges or gradually disintegrate. They may appear as a broad, hyaline, amorphous band at the periphery of the alveolus. The elastic fibers, as shown by Weigert's stain, also swell and undergo fragmentation as the process goes on. Many polymorphonuclears appear in the capillaries and migrate into the air vesicles, and with them comes an ever increasing number of large, ovoid, or slightly polygonal cells, which contain carbon and carmine at first to a slight, later to a marked degree (Fig. 1). They are in no way different from the mononuclear migratory cells described under the control experiment; at first they contain little or no ink, as they were presumably free in the alveoli before it was administered, but later they take it up in the vessels and appear in the alveoli with many granules in their cytoplasm, as can be seen in the photomicrographs.

As early as 5 hours after injection one can see marked proliferation of the endothelial cells of the capillary wall (better seen after 24 hours as in Fig. 2). Mitotic figures and ink globules are easily found. The endothelium is swollen and the cells are often in layers, or form nodal thickenings. Many figures of eight nuclei are present, and some swollen and distorted forms can be demonstrated, suggesting amitosis. The epithelium, on the other hand, does not show regenerative activity until the 3rd or 4th day, when it begins to proliferate, chiefly in the neighborhood of the bronchioles (Fig. 3). Here too, nodal heaps of cells occur, but they lie on the epithelial surface and project into the alveoli. Their cells are paler, more reticular, and contain no carbon, though they may take up a few bacilli. After 2 or 3 days the epithelium forms the familiar sheets of cuboidal cells on the alveolar wall and shows less activity. These sheets not infrequently grow over and enclose the masses of endothelial migratory cells which form in the alveolar spaces, sometimes almost entirely covering them with an epithelial envelope.

By the 4th day tubercles are found in the interstitial tissue, at the site of small collections of lymphoid cells (Fig. 4). They are composed of mononuclear cells containing ink, a few with the morphology of these but without carbon, and a few of the lymphocytes already present in the nodules, with a scattering of polymorphonuclears which have been attracted to the lesion. A day or so later the mononuclears in the alveolar spaces, which are now present in large numbers, fuse to form anastomosing masses of tubercles and syncytia, or giant cells (Fig. 5). In the series resulting from the injection of the coarser suspension of bacilli these areas are isolated and patchy (Fig. 6); in those in which the finer suspension was used the entire section is solid with the mass of mononuclear migrants, in many of which are mitotic figures, as in Fig. 3, showing that they are still actively growing. Numerous syncytia, commonly miscalled giant cells, are present and contain ink, carmine granules, and tubercle bacilli (Fig. 5). They first appear on the 3rd day after injection. The mononuclear endothelial cells of the exudate are often loaded with tubercle bacilli, as well as with ink and carmine (Fig. 6). While the intraalveolar tubercles have a predominatingly red color, that of the interstitial variety is black. On the 5th day these two types tend to fuse with one another.

including all the tissue between them, and large conglomerate areas result, which represent localized foci of tuberculous bronchopneumonia. As will be seen in Fig. 7, the fibroblasts that are included in these fusions begin to grow through the masses of endothelial cells and form interlacing bands of young connective tissue.

The reaction where a widespread lobar type of pneumonia resulted is essentially the same; there are more polymorphonuclears in the exudate and many erythrocytes are present. Both of these, with the endothelial mononuclears, fill whole sections of a lobe and, becoming caseous, form the exudate which gives this type of tuberculous pneumonia its name. The gelatinous exudate also appears and is found in alveoli where there is less active inflammation and cellular reaction and whose lymphatics are always widely dilated. Undoubtedly this is formed from coagulated lymph and a certain amount of very delicate fibrin, which can be demonstrated by using phosphotungstic acid hematoxylin either as a fine network or in a finely granular form, almost always without cellular admixture. The destruction of the elastic fibers is most marked in this type of inflammation; the fibrillæ are broken into very short, curly structures, not unlike large bacilli in their appearance. Here, then, the process is more diffuse and acute than in the cases in which the bacilli were less finely and evenly suspended and hence apt to enter the lung tissue in clumps.

The large phagocytic endothelial cells can be found in the lymphatics in this experiment, as they were in the controls. They are recognizable by their carmine and by the fact that tubercle bacilli can be demonstrated in their cytoplasm. That they contain ink in this case would, alone, prove that they came originally from the vascular endothelium; but the presence in these cells of the carmine and bacilli, which were introduced intratracheally, indicates that the cells come from the alveolar spaces. Hence we have pigmentary evidence that they have originated in the capillary walls, migrated to the air spaces, taken up bacilli and carmine, and, reentering the lymphatics, have carried these to the lymph nodes. Why they do so is not evident. This throws an interesting light on the spread of tuberculosis by means of migrating phagocytes; moreover, it furnishes additional evidence as to the endothelial origin of these cells, for such behavior is not characteristic of epithelium as we usually understand it.

The lymph nodes of the peribronchial lung tissue, as well as those of the mediastinal peribronchial group, show progressive increase in reticular cells and decrease in lymphocytes and lymphoblasts; small tubercles develop in the pulmonary nodes on about the 6th day. Most of these tubercles seem to be caused by an extension of the infection from tubercles in their immediate neighborhood. If bacilli carried to the nodes by phagocytes cause a development of tubercles in them, such a process would take place later, as there is little direct evidence thereof in this experiment, which represents a lapse of but 7 days after the introduction of the bacilli. Lymphocytes appear in the tubercles on about the 6th day; they seem to play no part in the primary formation of these lesions.

Another series of experiments is now in progress, a full report of which will appear in the near future. In these each rabbit receives in an ear vein 0.01 mg. of tubercle bacilli, of the same strain as that used in half the animals of the foregoing experiment. Niagara blue 3 B, an American form of trypan blue, is given intraperitoneally and Higgins' ink intravenously until the animals are vitally stained. The tubercles that result in the lung are of the miliary variety, almost all interstitial in location, apparently originating in, or near blood vessels and lymphoid tissue in the vicinity. They are deeply stained with ink and do not appear to take on much of the blue. There is little or no intraalveolar exudate or tubercle formation, in sharp contrast to the preceding experiment, in which the intratracheal infection resulted in striking exudation into the alveoli. In some places where the tubercles have become well developed and caseation is in an advanced stage, there is a spread to the alveoli (7 weeks), which gives rise to a reaction slighter than, but similar to that in the air-borne infection. This will be described in detail in the next paper, in connection with the lesions in the liver, spleen, kidney, etc., which, of necessity, result from a generalized hematogenous infection with tubercle bacilli. There is no doubt that there is a striking difference between the two types of infection when experimentally produced. All the hematogenous tubercles are composed of cells which contain more ink than do those found in the tubercles in the intratracheal infections. If the ink is administered for a while and the injections are then stopped, the tubercles are found to be deeply pigmented in

the older portions, while the newer zones of these lesions and the more recently formed lesions show very little carbon. For this reason it is necessary to continue the ink injections at least twice a week until the animal is killed.

Comparing the results of the experiments on the two types of infection, we find that the interstitially formed tubercles of the air-borne type compare well with those that predominate in the hematogenous type; they probably originate in the lymphatics in the first case and hence present this striking similarity. In the experiments on hematogenous infection we have no difficulty in tracing the origin of the epithelioid cell to the vascular endothelium; one can find tubercles produced as crescents in the lumina of medium sized capillaries, composed of cells that are so loaded with ink that their architecture is almost obscured. These tubercles can also be found in intratracheally infected series, but are more difficult of demonstration, for in this case we are dealing with secondary lesions, caused by bacteria which have escaped from the air vesicles, a condition which does not occur very frequently. The marked indifference of the epithelium to the hematogenous infection is another point against the theory that this tissue is the primary respondent to the tubercle bacillus.

It will be seen from what has been said that the findings in these experiments do not differ materially from those of Wechsberg, Watanabe, or Sewell; owing to the intravenous use of a colloidal suspension of India ink, however, the conclusions are decidedly different from those of the last two observers. If this material is not specific for the vascular endothelium, or at least selective enough in its action to be considered practically specific, this work must be discarded and new lines of approach devised before the question can be settled definitively. It appears that a specific agent has finally been found for marking the endothelial cell *in situ* and following it through its wanderings.

SUMMARY.

1. The injection of a colloidal suspension, or sol, of carbon into the veins of a living animal, as recommended by McJunkin, furnishes an apparently reliable means of tracing the so called epithelioid cell of the pulmonary tubercle from its origin in the vascular endothelium to the lesion.

2. Experimental tubercles are formed in the lung, as in the liver, primarily by cells originating in the capillary endothelium. These cells are probably present in small numbers in the normal lung, lying free both in the alveolar wall and the air vesicles. In response to infection they proliferate in the capillary walls in the vicinity of the invading organisms, migrate in steadily increasing numbers, and, arriving at the site of the infection, further multiply and to some extent fuse to form the syncytia known as giant cells.

3. The epithelial cell takes no active part in the process; its proliferation tends to repair denuded surfaces and is regenerative rather than combative or phagocytic in nature. This cell is free from carbon and stains only diffusely with carmine, in contradistinction to the endothelial cell which readily takes up both pigments in granular form.

4. The cells of endothelial origin not only phagocytose tubercle bacilli, but carry them into the tissues, for example into lymph nodes, by way of the lymphatics, or into other lung lobules by way of the air passages, in which they are readily demonstrable.

I am indebted to Professor J. Lewis Bremer for his kind permission to use the photomicrographic apparatus of the Department of Anatomy, and to his staff for their assistance and cooperation in its use.

BIBLIOGRAPHY.

Bremer, J. L., *Am. J. Anat.*, 1904, iii, 67.

Briscoe, cited by Sewell.

Ellenberger, W., Handbuch der vergleichenden mikroskopischen Anatomie der Haustiere, Berlin, 1906–11, iii.

Foot, N. C., *J. Med. Research*, 1919, xl, 352.

Foot, N. C., *J. Exp. Med.*, 1920, xxxii, 513.

McJunkin, F. A., *Arch. Int. Med.*, 1918, xxi, 59.

McJunkin, F. A., *Am. J. Anat.*, 1919, xxv, 27.

Minot, C. S., Human embryology, New York, 1892.

Sewell, W. T., *J. Path. and Bact.*, 1918–19, xxii, 40.

Slavjansky, K., *Virchows Arch. path. Anat.*, 1869, xlviii, 326.

Stöhr, P., Lehrbuch der Histologie und der mikroskopischen Anatomie des Menschen, Jena, 16th edition, 1915.

Tchistovitch, N., *Ann. Inst. Pasteur*, 1889, iii, 337.

Watanabe, K., *Beitr. path. Anat. u. allg. Path.*, 1902, xxxi, 367.

Wechsberg, F., *Beitr. path. Anat. u. allg. Path.*, 1901, xxix, 203.

EXPLANATION OF PLATES.

Plate 87.

Fig. 1. 5 hours after injection with tubercle bacilli. Note the large endothelial migratory cells in the alveolar spaces and the dilatation of the capillaries and lymphatics. × 1,000.[1]

Fig. 2. 24 hours after injection. The alveolar walls are thickened; polymorphonuclears are more numerous. × 1,000.

Fig. 3. 4 days after injection. Epithelial proliferation and partial fusion of the migrated endothelial cells, one of which shows a diaster. Carbon is not present in the epithelium, but is clearly seen in the intraalveolar mass of cells. × 1,000.

Fig. 4. 4 days after injection. The interstitial type of tubercle, very much richer in carbon than the intraalveolar form. × 1,000.

Plate 88.

Fig. 5. 6 days after injection. Three well developed syncytia. Note the contained carbon particles. Several smaller cells with carbon are present. × 438.

Fig. 6. 1 week after injection. Part of an intraalveolar tubercle, showing carbon in the cells, as well as tubercle bacilli. × 657.

Fig. 7. 1 week after injection. Low power photomicrograph of an entire lesion, of the conglomerate, intraalveolar type. The streaks are groups of fibroblasts; organization is under way. × 163.

---

[1] The figures of magnification are approximate but fairly accurate.

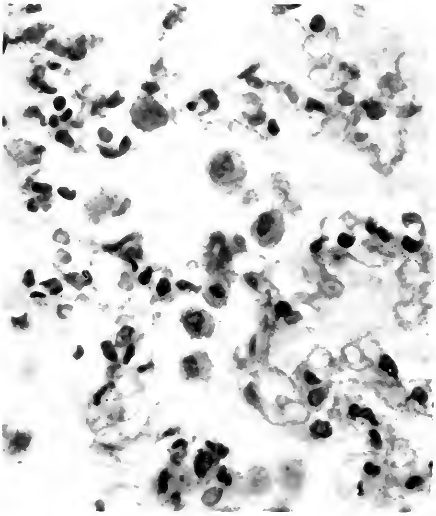


FIG. 1.

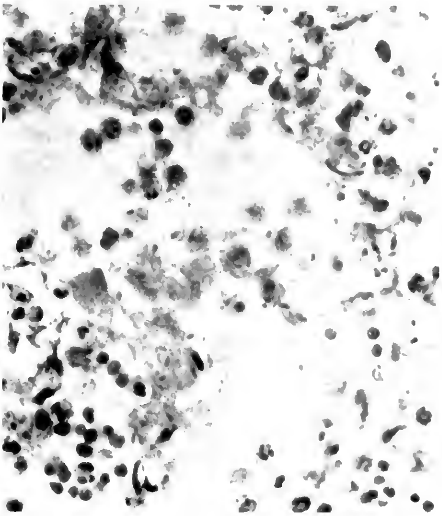


FIG. 2.

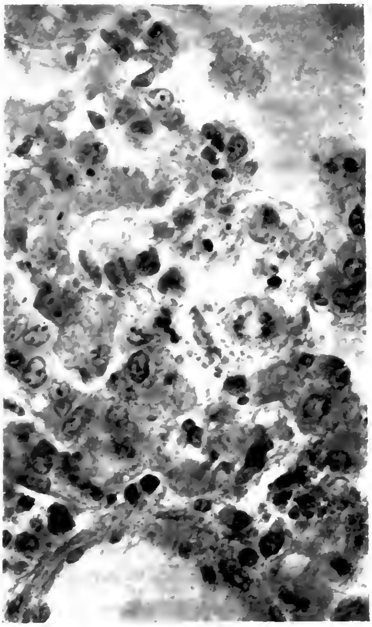


FIG. 3.

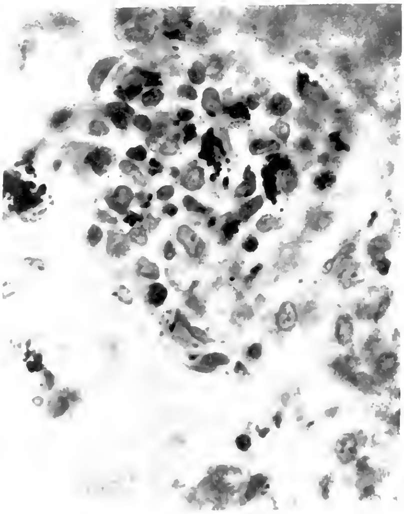


FIG. 4.

(Foot: Endothelial reactions. III.)

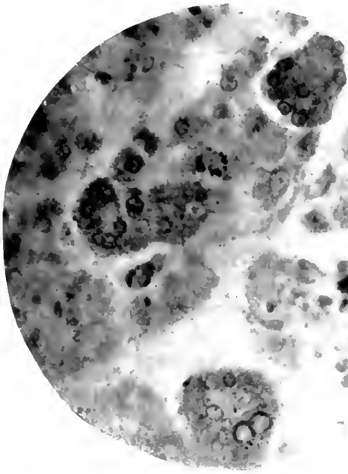


FIG. 5.

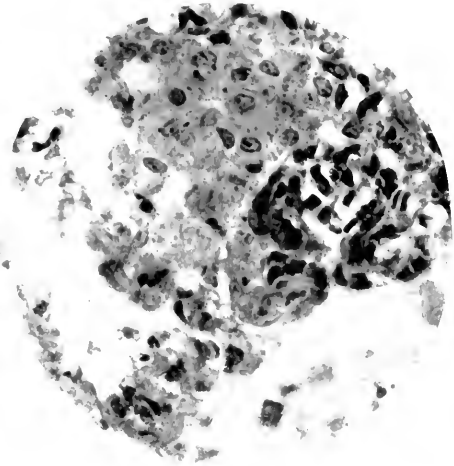


FIG. 6.

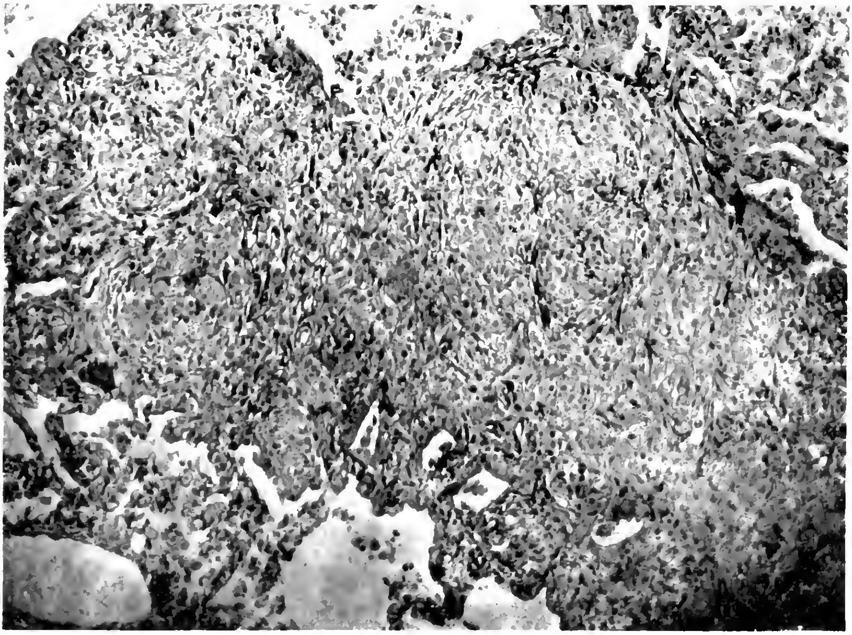


FIG. 7.

(Foot: Endothelial reactions. III.)

STUDIES ON THE ENZYMES OF PNEUMOCOCCUS.

I. Proteolytic Enzymes.

By O. T. AVERY, M.D., and GLENN E. CULLEN, Ph.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 11, 1920.)

Study of the biology of pneumococcus has led to a knowledge of certain biochemical characters which are common to the species as a whole, and to the recognition of fixed antigenic properties which serve to distinguish type differences within the species. The antigenic properties are inherent in the specificity of the bacterial protein and are detectable only by serologic reactions by means of which type relationships are recognized. The biochemical characters, on the other hand, are possessed in common by most pneumococci regardless of type differences, and are intimately associated with the life processes of the organism. These metabolic functions, upon which life of the cell depends, are in most instances referable to enzyme action. The presence or absence of a particular enzyme or group of enzymes determines largely the cellular activities of a microorganism. With the hope, therefore, of acquiring a better understanding of the way in which pneumococci adapt themselves to different environments, both in satisfying their nutritional needs and in exhibiting their invasive properties, the present study on the nature of the intracellular enzymes of this organism has been undertaken.

In the isolation and study of the enzymes of pneumococcus, apart from the living cell to which they are so intimately bound, use has been made of the fact that this organism undergoes rapid and complete solution in the presence of bile. Moreover, bile dissolves the bacterial cell with little or no change in the specific antigenic substance and with little or no injury to other demonstrable intracellular substances, such as the endohemotoxin. By dissolving the pneumococcus in bile and testing the cell-free solution on suitable substrates, enzymes are readily demonstrable. These enzymes have been found

to possess to a remarkable degree the power of actively hydrolyzing peptones to simpler peptides and amino-acids, of converting carbohydrates to simpler products, and of splitting esters to fatty acids. In demonstrating carbohydrate cleavage, however, bile was found to inhibit completely the hydrolysis of sucrose and starch, and another method of preparing the enzyme solution was necessary. This point will be emphasized in the paper on the intracellular invertase and amylase of pneumococcus (1).

The present paper concerns itself with the study of the proteolytic enzymes of pneumococcus. The intracellular nature of the enzymes, the influence of hydrogen ion concentration, the effect of age and concentration of the enzyme upon activity, and the relation of these enzymes to the virulence of the organism and to the mechanism of bile solubility will be discussed.

EXPERIMENTAL.

Bacteriological Methods.

Media.—The beef infusion broth containing 1 per cent peptone was prepared as previously described (2) except that (*a*) 2 gm. of dibasic phosphate per liter—anhydrous sodium phosphate or potassium phosphate—were used instead of 5 gm. per liter of sodium chloride, and (*b*) the medium was adjusted to a pH of 7.8.

Bile.—The ox bile used in preparing the pneumococcus enzyme solution was autoclaved, filtered, and again autoclaved as previously described (2).

Sterility Controls.—No antiseptics were used. The sterility of the enzyme solution was tested in broth and on blood agar plates. After addition of enzyme solution to the substrate, cultures of the mixtures were made by adding 0.1 cc. to 5 cc. of plain broth. In all the experiments recorded these controls remained sterile.

Chemical Methods.

Preparation of Substrate Solutions.—2 per cent solutions of peptone or protein were made in distilled water and the reaction was adjusted to the desired pH. The 2 per cent solution was then diluted with

an equal volume of 0.1 M phosphate solution of the desired pH. The final concentrations, unless otherwise stated, were therefore 1 per cent of substance in 0.05 M phosphate solution. The phosphate solutions were prepared from Merck's special reagents (Na_2HPO_4 and KH_2PO_4) according to Sörensen's (3) tables.

Sterilization.—Unless otherwise stated, sterilization was accomplished by autoclaving for 20 minutes at 15 pounds pressure.

Hydrogen Ion Concentration.—The pH values were usually determined colorimetrically, with the series of indicators outlined by Clark and Lubs (4). The solutions were diluted with two volumes of redistilled water, and the indicators used in such strength that one drop was required per 3 cc. of diluted solution. The readings were made by the comparator method (Walpole). Sörensen's standard phosphate and Walpole's (5) standard acetate solutions were used. These determinations were frequently checked by the electrometric method.

Nitrogen Determinations.—Total nitrogen determinations were made by the Kjeldahl method. Amino nitrogen was determined by Van Slyke's (6) nitrous acid method. With the peptone solution the determinations were made directly on 2 cc. samples, by means of the micro apparatus. Determinations of the amino nitrogen of the protein solutions were done by one of two methods: (*a*) 10 cc. samples were deaminized for 15 minutes in the large Van Slyke apparatus, and the nitrogen liberated was read in the micro burette calibrated to 0.002 cc.; and (*b*) the protein was precipitated with colloidal iron in the manner described by Van Slyke, Vinograd-Villchur, and Losee (7).

In determining the peptide nitrogen of the peptone solutions the peptides were split to amino-acids by acid hydrolysis (Van Slyke (8)). The peptide nitrogen was then calculated as the increase in amino nitrogen.

Action of Intracellular Enzymes of Pneumococcus on Peptone (Fairchild).

Experiment 1. (*a*) *Preparation of Enzyme.*—The washed bacterial residue from 2 liters of an 18 hour plain broth culture of Pneumococcus Type II (No. F 208) was taken up in 15 cc. of 33 per cent dilution of sterile bile (bile, 5 cc., + water, 10 cc.) and placed in the ice box over night. A portion of this bile solution of pneumococcus was inactivated by heat, as a control.

(*b*) *Preparation of Substrate.*—20 cc. portions of 1 per cent Fairchild's peptone in 0.05 M phosphate solution of various hydrogen ion concentrations were sterilized by the Arnold method on 3 successive days.

(*c*) *Sterility Control.*—No antiseptics were used. After the addition of enzyme solution to substrate, cultures of all digestive mixtures were made by adding 0.1 cc. of each to 5 cc. of plain broth. All cultures, including that of the bile solution of pneumococcus, were sterile.

The experiment was carried out as follows: Duplicate 20 cc. portions of the peptone substrate at reactions of pH 4.2, 5.3, 6.2, 7.0, and 7.6 were prepared. To one tube of each set, 1 cc. of enzyme solution was added, to the other 1 cc. of the inactivated enzyme solution. The tubes were then kept at 37°C. for 2 days. No antiseptic was used, sterility being maintained throughout by bacteriological technique. All cultural controls remained sterile, and after 48 hours the tubes were removed from the incubator for analysis. Duplicate amino-acid nitrogen determinations were made on 2 cc. samples by the Van Slyke method. The estimations of hydrogen ion concentration were determined colorimetrically on 5 cc. portions. The results are tabulated in Table I.

TABLE I.

Determination of Peptone-Splitting Activity (Fairchild's Peptone).

Tube No.	Final hydrogen ion concentration.		Amino nitrogen per 100 cc. of substrate.		
	Inactive.	Active.	Inactive.	Active.	Increase.
	pH	*pH*	*mg.*	*mg.*	*mg.*
1	4.2	4.2	27.3	27.2	
2	5.3	5.3	27.5	32.3	4.7
3	6.2	6.2	27.6	45.3	17.7
4	7.0	7.1	29.5	49.8	20.3
5	7.6	7.6	28.2	47.9	19.6
Peptone.			28.2		

Distribution of Nitrogen per 100 Cc. of Substrate.

		mg.
Total nitrogen		107
Amino "	before hydrolysis	27.5
" "	after "	78.6
Peptide "	therefore equals	51.1

Analysis of Experiment.

The increase of 20.3 mg. of amino nitrogen per 100 cc. of substrate shows that 40 per cent of peptide nitrogen was split to free amino nitrogen.

In order to determine the proportion of the peptide that was digested, a nitrogen partition was done on the peptone solution. Total nitrogen on 2 cc. portions was determined by the Kjeldahl method. Peptide nitrogen[1] was computed as the difference between total amino-acid nitrogen before and after hydrolysis.

Action of Intracellular Enzymes of Pneumococcus on Peptone (Witte).

Experiment 2. (*a*) *Preparation of Enzyme.*—This was the same as in Experiment 1, except that the bacterial residue from 2 liters of Pneumococcus Type II (No. F 208) was dissolved in 15 cc. of 25 per cent solution of sterile bile in water.

(*b*) *Preparation of Substrate.*—The substrate was prepared as in Experiment 1, except that Witte's peptone was used instead of Fairchild's preparation.

(*c*) *Sterility Control.*—No antiseptic was used. All tubes including cultural control of the enzyme solution were proved sterile by subcultures as in the preceding experiment.

In Experiment 2 the peptone-splitting action of pneumococcus enzyme was tested on Witte's peptone. The experimental technique was the same as that described in the preceding protocol, except for the substitution of 1 per cent Witte's peptone for the Fairchild preparation. The Witte peptone substrate was analyzed for total nitrogen, and peptide nitrogen in the same manner. The results are given in Table II.

It is evident from Experiments 1 and 2 that pneumococcus contains within its cell an enzyme or enzymes capable of hydrolyzing peptides into amino-acids or simpler peptides. From 26 to 40 per cent of the peptide nitrogen present in the peptone substrate was split to amino nitrogen. The term "peptone" solution is used to indicate the mixtures of partially hydrolyzed protein products which are known commercially as "peptones." The considerable data available as to the chemical nature of these peptones show them to be mixtures of protein products of varying degrees of complexity. The amount of pep-

[1] By peptide nitrogen is meant nitrogen found in the peptide linkings, the $-CO-NH-$ groups that link the different amino-acids together in peptides, proteins, or intermediate products. The process of hydrolysis consists in the splitting of these peptide groups, from each of which is generated a carboxyl group and an amino group. Thus

$$RNH - CO - R^1 + H_2O = RNH_2 + R^1COOH$$

For further discussion of this point see Van Slyke, D. D., *Arch. Int. Med.*, 1917, xix, 56.

tide hydrolyzed was greater in the experiment in which Fairchild's peptone was used. The proportion of preformed amino nitrogen to total nitrogen in this preparation was greater than in the sample of Witte's peptone, indicating that, on the average, Fairchild's peptone consists of simpler intermediate protein digestion products than Witte's. This may be the reason that further digestion by the enzyme proceeded more rapidly in the Fairchild product, the enzyme attacking the simpler peptides of the preparation the more readily.

TABLE II.

Determination of Peptone-Splitting Activity (Witte's Peptone).

Tube No.	Final hydrogen ion concentration.		Amino nitrogen per 100 cc. of substrate.		
	Inactive.	Active.	Inactive.	Active.	Increase.
	pH	*pH*	*mg.*	*mg.*	*mg.*
1	4.4	4.4	15.5	15.6	
2	5.0	5.0	15.6	21.2	5.6
3	6.0	6.0	15.4	32.4	17.0
4	7.0	7.0	15.5	39.6	24.1
5	7.8	7.8	15.5	39.4	23.9

Distribution of Nitrogen per 100 Cc. of Substrate.

	mg.
Total nitrogen	148
Amino " before hydrolysis	15.5
" " after "	108
Peptide "	92.5

Analysis of Experiment.

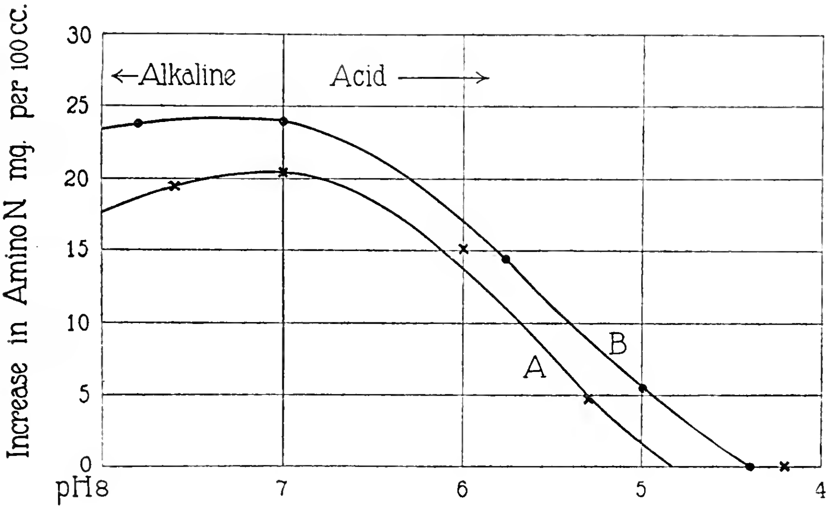
The increase of 24.1 mg. per 100 cc. of substrate shows that 26 per cent of peptide nitrogen was split to free amino nitrogen.

Effect of Hydrogen Ion Concentration on the Activity of Pneumococcus Peptonase.

Experiments 1 and 2 were planned to determine the relation of enzyme activity to hydrogen ion concentration. This relation is evident from the curves of Text-fig. 1, in which *A* represents Experiment 1 and *B* Experiment 2. The optimum activity of the peptone-splitting enzyme is from pH 7 to pH 7.8. With increase in acidity the

activity of the enzyme is increasingly retarded until complete inhibition results at pH 4.5.

These facts, that about 30 to 40 per cent of the peptide is hydrolyzed and that the optimum zone for activity of the enzyme is between pH 7 and 7.8, place it in the erepsin class of enzymes. Moreover, the fact that the curve of acid inhibition proceeds in a straight line to complete inhibition at a pH of about 4.5 indicates that the enzyme preparation used contains no pepsin and is, therefore, not a complex



Text-Fig. 1. Influence of hydrogen ion concentration on the activity of pneumococcus peptonase. Curve *A*, Experiment 1; Curve *B*, Experiment 2.

of erepsin- and pepsin-like enzymes. Since it seemed desirable to maintain an open mind on the question as to whether the enzymotic action in these experiments is more closely allied to trypsin or erepsin, and in view of its relative rate of action on native protein, the term "peptonase" has been used throughout the remainder of this report. It is significant that the optimum reaction zone for the intracellular peptolytic enzyme corresponds with the optimum for growth of pneumococcus (9).

Use of Sodium Choleate in Demonstrating the Intracellular Enzymes of Pneumococcus.

It is well known that pneumococci undergo solution in the presence of bile salts as completely as in the presence of bile itself. In order to determine whether the substitution of sodium choleate for bile in dissolving the organisms exerted any influence on the activity of the intracellular enzymes the following experiment was carried out.

Experiment 3. (*a*) *Preparation of Enzyme.*—Pneumococcus Type I (No. G_2) was grown in 2 liters of plain broth for 18 hours at 37°C. The bacteria were removed by centrifugation, washed once in sterile isotonic salt solution, and suspended in 10 cc. of 5 per cent solution of sodium choleate. After 5 hours in a water bath at 37°C., the resultant solution of pneumococci was diluted with an equal volume of sterile distilled water. One portion of this solution was inactivated by heat and both were tested for activity in a peptone substrate.

(*b*) *Preparation of Substrate.*—20 cc. portions of 1 per cent Fairchild's peptone in 0.05 M phosphate solution were adjusted to the various hydrogen ion concentrations and sterilized by the Arnold method on 3 successive days.

This experiment was conducted in exactly the same manner as Experiment 1.

TABLE III.

Determination of Peptone-Splitting Activity of a Solution of Pneumococcus Obtained by the Use of Sodium Choleate.

Tube No.	pH	Amino nitrogen per 100 cc. of substrate.		
		Inactive.	Active.	Increase.
		mg.	*mg.*	*mg.*
1	5.0	37.9	42.9	5.0
2	5.4	37.9	50.7	12.8
3	5.8	38.7	53.4	14.7
4	6.2	40.4	55.2	14.8
5	6.6	41.6	59.2	17.6
6	7.0	42.2	60.3	18.1
7	7.4	39.6	58.9	19.3
8	7.8	40.7	58.3	17.6

From the data presented in Table III it is evident that when pneumococci are dissolved by sodium choleate there is liberated from the cell a peptone-splitting enzyme in the same manner as when solution of the organism is effected by the action of bile. It has also been

found that enzyme solutions obtained by disintegration of pneumococcus cells without the presence of bile or bile salts, by methods described in a succeeding paper (1), exhibit comparable activity. Therefore, bile salts are not essential to the action of the enzyme.

Effect of Age on the Activity of the Intracellular Peptonase of Pneumococcus.

Experiment 4. (*a*) *Preparation of Enzyme.*—The technique of preparing the enzyme solution in this experiment was similar to that described in preceding protocols, except that the bacterial residue from 2 liters of broth culture of Pneumococcus Type II (No. F 208) was dissolved directly in 10 cc. of undiluted bile. After 2 hours in the water bath at 37°C. and 1 hour at room temperature, the enzyme solution was stored in the ice box, and at intervals up to 43 days portions were removed and tested for activity.

(*b*) *Preparation of Substrate.*—1 per cent of Fairchild's peptone in $\frac{M}{30}$ phosphate solution of pH 7 was prepared and sterilized in the autoclave at 15 pounds pressure for 20 minutes.

(*c*) *Sterility Control.*—No antiseptics were used. Cultures of the original enzyme solution and each digestion mixture at the time of carrying out the several tests proved sterile.

The results are presented in Table IV.

TABLE IV.

Age Stability of Endopeptonase.

Enzyme solution kept at about 4°C. 1 cc. added to 20 cc. of peptone (Fairchild), pH 7. 24 hours at 37°C.

Age.	Amino nitrogen per 100 cc. of substrate.		
	Inactive.	Active.	Increase.
days	*mg.*	*mg.*	*mg.*
0	37.4	58.8	21.4
1	37.4	59.7	22.3
2	40.8	62.6	21.8
6	37.4	53.6	16.2
20	40.8	54.4	13.6
43	23.4	32.1	8.7

The length of time an enzyme may remain active is dependent upon the conditions of its preservation. The age stability of an

enzyme in dried form is greater than that of the same enzyme in solution. In the present instance, the intracellular peptonase of pneumococcus dissolved in undiluted ox bile retained about 40 per cent of its activity for over 6 weeks.

Relation of Virulence of Pneumococcus to Enzyme Activity.

Experiment 5. (*a*) *Preparation of Enzyme.*—Plain broth cultures (1,500 cc.) of a virulent and avirulent strain of Pneumococcus Type II (No. F 208) were centrifuged; the bacterial sediment was washed once in sterile isotonic salt solution, then dissolved in 15 cc. of undiluted bile, and held in the ice box over night. Portions of the enzyme solutions were inactivated by heating in the autoclave at 15 pounds pressure for 20 minutes.

TABLE V.

Influence of Virulence of Pneumococcus upon the Activity of the Intracellular Peptonase.

2 cc. of enzyme solution added to 20 cc. of peptone solution, pH 7. Incubated at 37°C. for 24 hours.

Pneumococcus Type II.	Minimum fatal dose.	Amino nitrogen per 100 cc. of substrate.		
		Inactive.	Active.	Increase.
	cc.	*mg.*	*mg.*	*mg.*
No. F 208 A	0.000001	39.8	68.6	28.8
" F 208 "B"	Greater than 1	39.2	67.8	28.6

(*b*) *Preparation of Substrate.*—1 per cent peptone (Fairchild) in $\frac{M}{30}$ phosphate solution, pH 7, was sterilized in the autoclave.

(*c*) *Sterility Control.*—No antiseptics were used Each tube in the experiment was tested for sterility by subculture and yielded no growth.

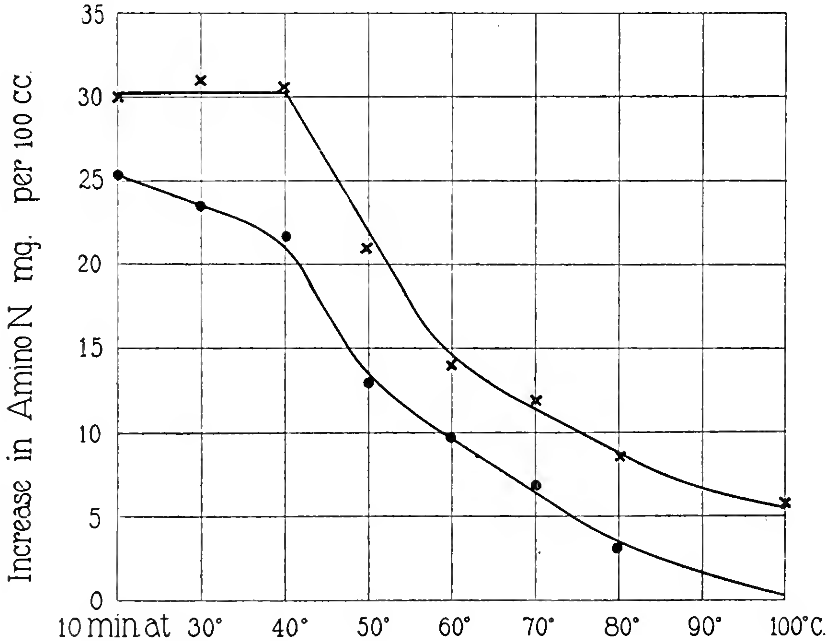
The development of a technique for the demonstration of endoenzymes made it possible to submit to experimental proof the question whether differences in virulence are in any way related to the activity of the intracellular enzymes. For this purpose a strain of Pneumococcus Type II (No. F 208) was chosen. This organism was originally isolated from the blood of a patient suffering from lobar pneumonia. The virulence of the strain, maintained by animal passage, was such that 0.000001 cc. of broth culture injected intraperitoneally into white mice proved fatal in 24 to 48 hours. A subculture of the same strain, the virulence of which had been attenuated by cultural methods, failed to kill mice in doses of 1 cc. Enzyme solutions from comparable amounts of bacteria were prepared from the virulent and avirulent cultures of this strain. The respective

enzyme preparations were tested for peptonase action by adding 2 cc. of each to substrates of 20 cc. of 1 per cent peptone solution adjusted by phosphates to pH 7. After 24 hours at 37°C. the degree of enzyme action was determined by measuring the increase of amino nitrogen as indicated in Table V.

From Table V it appears that loss of virulence is not associated with a corresponding loss of enzymotic activity. Under the conditions of this experiment at least, the amount of hydrolysis of peptone by the endoenzymes of the avirulent strain was equivalent to that of the virulent organism.

Effect of Heat on the Intracellular Peptonase of Pneumococcus.

Sensitiveness to heat is a biologic character of all enzymes. In determining the influence of heat upon dissolved enzymes, the degree of temperature, the length of exposure, and the reaction of the solu-



TEXT-FIG. 2. Heat stability of intracellular peptonase of pneumococcus. The lower curve represents the results after 24 hours incubation at 37°C., the upper curve after 48 hours at 37°C.

tion are closely interrelated. The optimum reaction for activity of the endopeptonase of pneumococcus, pH 7.4, and an exposure of 10 minutes were arbitrarily chosen, and the temperature alone was varied as shown in Text-fig. 2.

Experiment 6. (*a*) *Preparation of Enzyme.*—The bacterial residue from 4 liters of plain broth culture of Pneumococcus Type II (No. F 208) was dissolved in 20 cc. of sterile ox bile and held in the ice box over night.

(*b*) *Preparation of Substrate.*—1 per cent peptone solution (Fairchild) in 0.05 M phosphate solution adjusted to pH 7.4 was sterilized in the autoclave.

(*c*) *Sterility Control.*—Sterility was proved by subculture from each tube.

1 cc. of the enzyme solution with a pH of about 7.4 was placed in each of eight sterile tubes, and these in turn were immersed in water baths at 30°, 40°, 50°, 60°, 70°, 80°, 90°, and 100°C., respectively, for exactly 10 minutes. On removal the tubes were immediately cooled, and to each were added 10 cc. of the sterile substrate. After 24 and 48 hours incubation at 37°C. samples were removed for analysis. The results are plotted in Text-fig. 2. The heat sensitiveness of the enzyme manifests itself in a progressive loss of activity after exposure for 10 minutes to increasing temperatures, until at 100°C. little or no activity remains.

Effect of Concentration of Enzyme on the Activity of the Intracellular Peptonase of Pneumococcus.

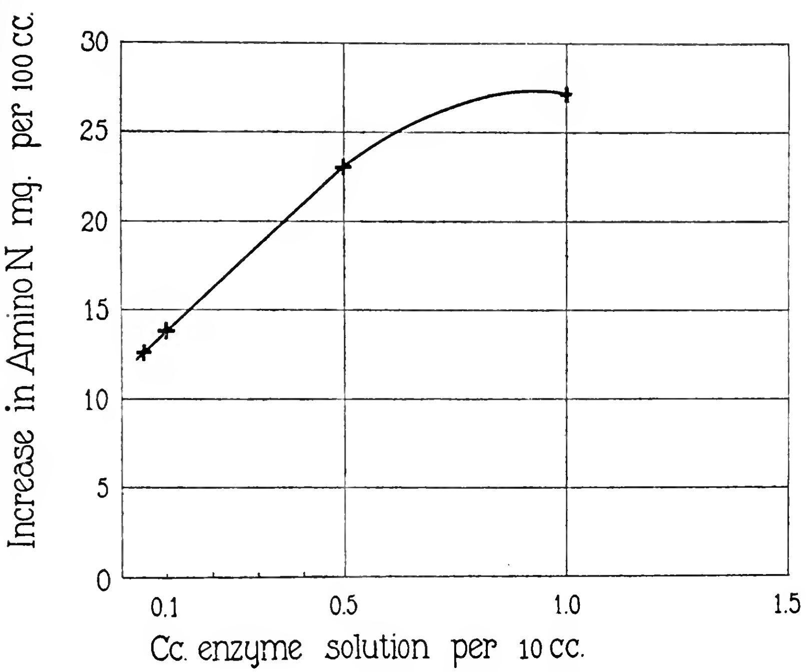
Experiment 7.—The same preparation of enzyme and substrate used in Experiment 6 was employed in this test, a bile solution of Pneumococcus Type II and a 1 per cent solution of peptone (Fairchild's) in 0.05 M phosphate mixture, pH 7.4.

The dissolved enzyme was diluted with bile so that 1 cc. of the final solution added to 10 cc. of peptone substrate contained 0.05, 0.1, 0.5, and 1 cc. respectively of the original enzyme solution. The results are plotted in Text-fig. 3.

For a more exact study of the dynamics of this enzyme it would be desirable to repeat this experiment with shorter digestion periods and smaller amounts of enzyme. It is evident, however, that under given conditions the rate of hydrolysis is proportional to the concentration of enzyme.

Occurrence of Enzymes in Culture Filtrates of Pneumococcus.

The experiments thus far have dealt entirely with intracellular enzymes. It was considered probable that the culture medium itself



TEXT-FIG. 3. Effect of concentration of enzyme on the activity of the intracellular peptonase of pneumococcus.

would contain similar enzymes which had either diffused out from the cell during growth or had been liberated by disintegration of the organisms in the culture fluid. In order to determine the validity of this assumption, the following experiment was undertaken.

Experiment 8. (*a*) *Preparation of Filtrate.*—100 cc. of an 18 hour plain broth culture of Pneumococcus Type II (No. F 208) was filtered through a Berkefeld candle N. The filtrate, pH 7, was tested for sterility by adding 5 cc. to 100 cc. of fresh broth and incubating at 37°C. In testing the sterility of culture filtrates of pneumococcus it is not sufficient merely to incubate the filtrate itself, but it is essential to inoculate fresh broth. This is probably due to the fact, recorded in a previous paper (Avery and Cullen (10)), that further growth cannot be initiated in the filtrates of a plain broth culture of pneumococcus even though the reaction is readjusted to the optimum hydrogen ion concentration. A portion of the enzyme-containing filtrate was inactivated by heat, to serve as a control.

(*b*) *Preparation of Substrates.*—These consisted of the sterile culture filtrate and a 1 per cent solution of peptone (Fairchild) in phosphate mixture adjusted

to pH 7, the same hydrogen ion concentration as the filtrate. The peptone solution was sterilized in the autoclave.

In determining the presence of enzyme in culture filtrates of pneumococcus the amount of cleavage was determined, (*a*) as the result of further action of the enzyme on the peptones present in the broth filtrates and (*b*) as the result of this action plus the action on additional peptones. This was accomplished by the procedure indicated in Table VI, three tubes of substrate being used, in all of which the final volume was 20 cc. The first tube contained 10 cc. of filtrate and 10 cc. of sterile water, the second, 10 cc. of filtrate and 10 cc. of 1 per cent peptone solution, and the third (the control), 10 cc. of 1 per cent peptone solution plus 10 cc. of sterile water. The determinations of amino nitrogen in the digestion mixtures before and after incubation are given in Table VI.

TABLE VI.

Presence of Peptonase in Culture Filtrate of Pneumococcus.

Tube No.	Sterile filtrate unheated; pH 7.	1 per cent peptone solution; pH 7.	Water.	Amino nitrogen per 100 cc. of final solution.		
				Before digestion.	After digestion.	Increase.
	cc.	*cc.*	*cc.*	*mg.*	*mg.*	*mg.*
1	10		10	54.9	73.4	18.5
2	10		10	54.7	73.0	18.3
3		10	10	24.0	24.0	
4		10	10	24.0	24.0	
5	10	10		78.9	106.8	27.9
6	10	10		79.0	106.8	27.8

Analysis of Table VI.

Increase in amino nitrogen in filtrate alone......... 18.4 mg. per 100 cc.
" " " " " " + peptone.... 27.9 " " 100 "
" " " " due to action of enzyme
on added peptone............................ 9.4 " " 100 "

From Table VI it is clear that under the conditions of the experiment the bacteria-free filtrate contained an active enzyme which continued to hydrolyze the excess of available peptide in the medium and in addition attacked the added peptone.

The demonstration of the presence of an enzyme in culture filtrates of pneumococcus after 18 hours incubation does not necessarily imply that the enzyme is a true secretory product which is elaborated during

growth and given off into the medium in the manner of exotoxins. Under optimum cultural conditions pneumococcus reaches its maximum growth relatively early (Chesney (11)), after which involution and disintegration of the bacterial cells soon begin. During this later period, the intracellular hemolysin of pneumococcus, which cannot be detected free in the medium during the early phase of active growth, is also released from the disrupted cell and can be demonstrated in the culture fluid. If the peptonase is an endoenzyme, its absence in filtrates during the period of active growth should be demonstrable.

In order, therefore, to test for presence of enzyme in the culture fluid before cell death and disruption with consequent liberation of intracellular enzyme occurred, the following experiment was carried out.

200 cc. of plain broth, pH 7.8, were inoculated with 0.5 cc. of a 5 hour culture of Pneumococcus Type II (No. F 208). After 5 hours incubation marked growth was apparent and the acidity had increased to pH 7.5. The culture was then centrifuged and the supernatant fluid was filtered through a tested Berkefeld candle N. This filtrate was kept in the refrigerator until its sterility was proved by culture. The sterile filtrate was then tested for enzyme activity as in the preceding experiment.

It was found that during the phase of active growth the culture fluid freed from bacteria possessed no peptolytic activity. Moreover, in this same culture fluid the intracellular hemolysin, known to be liberated by cell disintegration, was likewise not demonstrable, but the soluble substance shown by Dochez and Avery (12) to be elaborated during the earliest phase of cell multiplication was detectable in considerable concentration. The occurrence, therefore, of peptolytic activity in autolyzing broth cultures and its absence in the culture fluid during the early phases of active growth make it evident that the peptonase is a true endoenzyme.

Effect of Exposure to Acid Reaction on the Intracellular Peptonase of Pneumococcus.

It has been observed that cultures of pneumococci grown in sugar-containing medium reach a final hydrogen ion concentration of about pH 5 to 5.2. At this point the organisms quickly succumb, the acidity

produced by their own metabolic processes being sufficient in itself to stop growth. Lord and Nye (13) have found that pneumococci will not live for more than a few hours in a solution the acidity of which is greater than pH 5.1. Experiments in this laboratory have shown that pneumococci subjected to an acidity of pH 4.5 to 5 for 2 hours are no longer soluble in bile. Even when these organisms are removed by centrifugation, washed, and resuspended in phosphate solution at a pH 7.8, they remain insoluble in bile. The question arose whether this reaction of pH 4.5 to 5, which corresponds to the acid death-point of the bacterial cells, is also fatal to the intracellular enzymes, and whether this fact is of any significance in the phenomenon of bile insolubility.

To determine whether acid treatment injured the endoenzymes of pneumococcus the following experiment was carried out.

Experiment 9.—1 cc. of the enzyme solution used in Experiment 6 was treated with 4 cc. of 0.1 M potassium acid phosphate solution of pH 4.5. The reaction of the resulting solution was pH 5. After 2 hours at 37°C. the solution was readjusted to pH 7.4 by the addition of 0.3 cc. of N sodium hydroxide (calculated and verified on a separate sample), and 5 cc. of a 2 per cent peptone solution of pH 7.4 were added. As a control on activity, the untreated active enzyme was added to the same peptone substrate in similar proportions. The results are given in Table VII.

TABLE VII.

Effect on Peptonase of Exposure to Acid Reaction.

Final concentration of enzyme was 1 cc. per 10 cc. of 1 per cent peptone in 0.1 M phosphate solution of pH 7.4. 42 hours at 37°C.

Treatment of enzyme.	Amino nitrogen per 100 cc. of substrate.		
	Inactive.	Active.	Increase.
	mg.	*mg.*	*mg.*
Enzyme solution previously kept at pH 7.8	31.2	58	26.8
" " " adjusted to pH 5.0 and after 2 hrs. at 37°C. readjusted to pH 7.4	31.2	58	26.8

The intracellular enzyme in solution suffered no loss of potency after being subjected for 2 hours to an acidity of pH 5, for upon readjustment to the optimum hydrogen ion concentration of pH 7.4, the

acid-treated enzyme exhibited an activity comparable to that of the untreated enzyme. The endopeptonase of pneumococcus is evidently little influenced by this reaction change. The bile insolubility of pneumococci at the acid death-point is, therefore, not associated with destruction of this enzyme, but is probably referable to coagulative changes in the cell protoplasm.

Action of the Endoenzymes of Pneumococcus on the Proteins, Casein, Gelatin, Albumin, and Fibrin.

The data presented in the preceding protocols establish the fact that there is present within the bacterial cell an enzyme, or enzymes, capable of hydrolyzing peptones into amino-acids, and that this enzyme-complex manifests its optimum activity in a slightly alkaline medium. Simultaneous experiments were carried out with the same enzyme preparations to determine their action on the proteins, casein, gelatin, fibrin, and albumin.

Preparation of Enzyme.—In these experiments the enzyme solutions were prepared in the manner described in Experiment 6.

Preparation of Substrates.—2 per cent gelatin, albumin (egg), and casein solutions were adjusted to pH 7.4 and diluted with an equal volume of 0.1 M phosphate solution of the same hydrogen ion concentration and then sterilized in the autoclave. The fibrin substrate D was prepared by adding 0.2 gm. of dried commercial fibrin to 10 cc. of 0.05 M phosphate solution of pH 7.4 and was sterilized in the autoclave. The fibrin substrate F was freshly prepared from 10 cc. portions of sterile oxalated rabbit plasma. Sterile calcium chloride was added and the fibrin clot was washed in sterile salt solution and transferred to 10 cc. of sterile 0.05 M phosphate solution of pH 7.4.

Experiment 10.—To 10 cc. portions of the sterile protein solution 1 cc. of enzyme solution was added, and the mixture placed at 37°C. for 5 to 7 days. The degree of digestion was determined as in the peptone experiments by amino nitrogen determination with one of the following procedures: (*a*) 10 cc. portions of the digestion mixtures were placed in the deaminizing bulb of the original large type of Van Slyke apparatus. 15 minutes were allowed for deamination and the nitrogen evolved was measured in a micro burette graduated to 0.002 cc. (*b*) After removal of the proteins by colloidal iron, amino nitrogen determinations were made in the manner described in preceding experiments. The results are presented in Table VIII.

Experiment 11.—In following the extent of digestion of the peptone solution control amino nitrogen determinations on active enzyme alone had shown that

TABLE VIII.

Proteolytic Action of Endoenzymes of Pneumococcus.

Protein.	Duration of digestion.	Method of analysis.	Amino nitrogen per 100 cc. of substrate.		
			Inactive.	Active.	Increase
	days		*mg.*	*mg.*	*mg.*
Casein.	5	(*b*)	1.9	6.3	4.4
"	12	(*b*)	1.3	3.4	2.1
Gelatin.	7	(*a*)	6.3	6.7	0.4
	12	(*b*)	2.8	3.5	0.7
Fibrin D.	5	(*a*)	2.5	4.0	1.5
" F.	31	(*b*)	4.2	7.1	2.9

the increase in amino nitrogen due to autolysis of the bacterial proteins was negligible. However, because of the small concentration of free amino nitrogen in the protein experiment more rigid control of this protein digestion was carried out. Duplicate 10 cc. portions of casein, albumin, and 0.05 M phosphate, all at pH 7.4, were prepared. To one tube of each pair 2 cc. of active enzyme were added, and to the other 2 cc. of heated enzyme. Tubes were incubated at 37°C. for 5 days. Each dilution was then diluted to 25 cc., 5 cc. portions were removed for total nitrogen determinations, and the remaining 20 cc. were freed from protein by precipitation with colloidal iron. The non-protein nitrogen of the filtrate and washings was determined by the Kjeldahl method. The results are presented in Table IX.

TABLE IX.

Proteolytic Action of Endoenzymes of Pneumococcus.

Proteolysis measured by increase in non-protein nitrogen. Digestion for 5 days at 37°C.

Solution.	Total nitrogen per 100 cc. of solution.	Non-protein nitrogen per 100 cc. of solution.			Protein digested.
		Inactive.	Active.	Increase.	
	mg.	*mg.*	*mg.*	*mg.*	*per cent*
Enzyme....................	25.7	14.8	16.8	2.0	20.0
Casein*....................	14.6	7.0	19.4	12.4	8.9
Albumin*..................	12.3	6.4	6.5		

* Protein figures corrected for nitrogen of enzyme solution added.

From Table IX it is evident that autolysis of the enzyme solution itself is occurring. This increase would, for the concentrations used in Table VIII, amount to about 0.5 mg. of amino nitrogen per 100 cc.; that is, the increase in the case of the gelatin experiment is due to the enzyme solution. The increase of 3 and 4 mg. in the case of casein and fibrin indicates a definite proteolysis.

The endoenzymes of pneumococcus are apparently able partially to hydrolyze the proteins, casein and fibrin, but not albumin or gelatin. This enzyme action on intact protein is distinctly less, however, than that which occurs in the presence of peptones.

DISCUSSION.

To present a critical review of the literature on the nature and action of bacterial enzymes would entail a task beyond the scope of this paper. Although extensive study of the enzymes of a large variety of different species of microorganisms has been made, comparatively little work has been done on the occurrence and character of the endoenzymes of pneumococcus.

Rosenow (14) demonstrated in extracts of virulent pneumococci and filtrates of broth cultures a proteolytic enzyme capable of hydrolyzing the proteins in meat broth, in ascites meat broth, and to a less extent the proteins of heated serum. He prepared extracts of pneumococci by suspending the bacteria from broth cultures in salt solution, adding ether, and allowing autolysis to proceed at 37°C. for 48 hours. The bacteria were removed by centrifugation or passage through a Berkefeld filter. Rosenow found that the degree of disintegration of the bacterial cells was directly proportional to the amount of proteolysis as measured by formol titration. He has shown further that the toxicity of broth, culture filtrates, and extracts of pneumococci is associated with proteolysis due to the dissolved enzymes.

Evidence is presented in this paper of the existence of proteolytic enzymes within the cell body of pneumococcus. This enzyme or group of enzymes can be isolated from the living cell by dissolving the organisms in bile or allowing them to cytolyze in phosphate solutions of pH 6.2. In the latter method alternate thawing and freezing of the bacterial suspension greatly facilitate the extraction process by disrupting the bacterial cells.[2] By these methods the intracellular

[2] See Avery and Cullen (1).

substances pass into solution in a medium in which they retain their activity for a considerable period. The proteolytic activity of these enzymes is manifest in their ability to hydrolyze, to some extent, intact protein and to split to a striking degree intermediate products, such as peptones, into simpler peptides and amino-acids. It has not been determined whether the two processes, proteolysis and peptolysis, are functions of the same enzyme or the result of the action of two separate enzymes.

It is evident that the action on the intact proteins, fibrin and casein, is distinctly less than on simpler substances such as occurs in peptone mixtures. For this reason the larger number of experiments has been carried out with a partially hydrolyzed protein, commonly known as peptone, as substrate. Because it exhibits its maximum activity in the further hydrolysis of peptide nitrogen, this enzyme is referred to as peptonase, a term indicative of its action on peptone.

The intracellular peptonase of pneumococcus hydrolyzes 30 to 40 per cent of the peptide nitrogen in peptone substrates to amino nitrogen. The peptonase activity of the bacterial substance is striking in its intensity. Weight for weight the substance hydrolyzes peptone several times as rapidly as the most active commercial samples of pancreatic preparations. The zone of its optimum activity is pH 7 to 7.8, similar to that of trypsin and erepsin, and corresponds to the optimum reaction for growth of pneumococcus. The absence of activity at a pH below 4.5 indicates the absence of pepsin. Bile salts, as well as bile itself, effect solution of pneumococci, and enzymes prepared by dissolving the cell bodies in solutions of sodium choleate manifest an equal degree of activity. The thermostability of the intracellular peptonase is greater than the heat resistance of pneumococcus. The enzyme is, however, sensitive to heat; an exposure of 10 minutes at 100°C. destroys its activity. Dissolved in undiluted ox bile the enzyme retained about 40 per cent of its activity over a period of 6 weeks. A direct proportionality has been shown to exist between the rate of hydrolysis and the concentration of the enzyme in the digestion mixture.

In bacteria-free filtrates of pneumococcus enzymes are demonstrable only when growth of the bacteria has progressed to the phase in which cell disintegration begins and liberation of the intracellular sub-

stances into the culture medium occurs. During the early stages of growth of pneumococcus, when the organisms are multiplying at their maximum rate and little or no cell death is occurring, enzymes cannot be detected in culture filtrates. This evidence indicates that the enzymes studied are intracellular in character and belong to the class known as endoenzymes.

As far as is known for bacteria, solubility in bile is peculiar to pneumococcus alone. The mechanism of this reaction is not fully understood. Pneumococci exposed to an acidity equivalent to or greater than pH 5 are not only rapidly killed but rendered completely bile-insoluble. The endoenzymes derived from pneumococcus, on the other hand, are little influenced in their subsequent activity by previous exposure for 2 hours to a reaction corresponding to the acid death-point of the bacterial cell. Similarly pneumococci rapidly succumb on short exposure to a temperature of 52°C. and the heat-killed organisms are no longer soluble in bile. Exposure of the proteolytic enzyme, however, to a temperature corresponding to the thermal death-point of pneumococcus, causes only slight retardation of its hydrolyzing power.

These facts, apart from their significance in a study of the nature of the endoenzymes, are of interest in interpreting the possible relation of these active intracellular substances to the mechanism of bile solubility of pneumococcus. From these limited observations, it does not appear likely that bile solubility is the result of the action of enzymes, of which bile serves as an activator, for agents, both chemical and physical, which render the cell insoluble in bile, exert in a similar concentration only slight inhibition on the intracellular enzymes.

Rosenow found that extracts of virulent pneumococci possessed the power to split foreign proteins such as those present in ascites meat broth, while extracts of non-virulent organisms showed no digestion. The observations recorded in this paper on the relation of virulence to enzyme activity of pneumococcus are too limited to warrant any final judgment. However, under the experimental conditions, loss of virulence of the organism was not associated with a corresponding loss of enzyme activity. Pneumococci with virulence differing by a ratio of 1,000,000 to 1 showed quantitatively identical proteolytic power. In further elucidation of this problem it would be of interest

not merely to compare differences in enzymic activity with variations in virulence of the same strain, but also to contrast the relative potency of enzyme preparations from pathogenic pneumococci of the disease-producing types with the activity of similar preparations from the more saprophytic varieties of little or no virulence.

SUMMARY.

1. Pneumococci contain an intracellular enzyme or enzymes which (*a*) hydrolyze to some extent intact protein and (*b*) hydrolyze with striking avidity peptones. The optimum reaction for hydrolysis is pH 7 to 7.8, which also represents the optimum for the growth of pneumococcus. For convenience the terms "protease" and "peptonase" have been used, but no assumption is made as to whether the two actions, proteolysis and peptolysis, are due to two separate enzymes or are two activities of the same enzyme.

2. Solutions of intracellular substance of comparable enzymic activity may be prepared by dissolving the bacteria in bile, in sodium choleate, or by mechanical and autolytic disintegration of the cell.

3. The rapidity with which peptone is hydrolyzed is proportional to the concentration of the enzyme.

4. Heating the enzyme for 10 minutes at 100°C. destroys its activity.

5. Increasing the acidity to pH 5, the acid death-point of pneumococcus, suspends activity but does not destroy the enzyme, for activity is restored by readjustment to pH 7.8.

6. Attenuation of virulence to 1/1,000,000 of the original virulence had no measurable quantitative effect on the enzyme activity.

BIBLIOGRAPHY.

1. Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1920, xxxii, 583.
2. Avery, O. T., Chickering, H. T., Cole, R., and Dochez, A. R., Acute lobar pneumonia. Prevention and serum treatment, Monograph of The Rockefeller Institute for Medical Research, No. 7, New York, 1917, 19.
3. Sörensen, S. P. L., *Ergebn. Physiol.*, 1912, xii, 393.
4. Clark, W. M., and Lubs, A. A., *J. Bact.*, 1917, ii, 7.
5. Walpole, G. S., *Biochem. Z.*, 1911, v, 207.

6. Van Slyke, D. D., *J. Biol. Chem.*, 1913–14, xvi, 121.
7. Van Slyke, D. D., Vinograd-Villchur, M., and Losee, J. R., *J. Biol. Chem.*, 1915, xxiii, 377.
8. Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 295.
9. Dernby, K. G., and Avery, O. T., *J. Exp. Med.*, 1918, xxviii, 345.
10. Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1919, xxx, 374.
11. Chesney, A. M., *J. Exp. Med.*, 1916, xxiv, 387.
12. Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1917, xxvi, 477.
13. Lord, F. T., and Nye, R. N., *J. Exp. Med.*, 1919, xxx, 389.
14. Rosenow, E. C., *J. Infect. Dis.*, 1912, xi, 286.

STUDIES ON THE ENZYMES OF PNEUMOCOCCUS.

II. Lipolytic Enzymes: Esterase.

By O. T. AVERY, M.D., and GLENN E. CULLEN, Ph.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 11, 1920.)

In the preceding paper (1) are recorded the facts so far obtained in a study of the proteolytic enzymes of pneumococcus. It has been shown that bile solutions of pneumococci, extracts obtained by disintegration of the organisms in phosphate mixtures, and sterile filtrates of autolyzing broth cultures possess the power to hydrolyze peptones and to a less extent certain intact proteins. In the present paper evidence will be presented that pneumococci possess also an endolipase of marked activity. The intracellular nature of this enzyme, the influence of age and hydrogen ion concentration on its activity, its thermostability, and relation to virulence and to the mechanism of bile solubility will be discussed.

In a monograph on bacterial enzymes, Fuhrmann (2) (1907) summarizes the work of earlier investigators on the occurrence of lipases in different species of microorganisms. In a review of the literature no reference has been found to work on the lipase of pneumococcus. Various methods for the demonstration of lipolytic activity of bacteria have been used. By a simple plate method, in which the zone of reaction about colonies of bacteria growing in the presence of certain test substances such as fat could be observed, Eijkman (3) detected lipolytic action in a number of organisms, including *Staphylococcus aureus*, *B. pyocyaneus*, *B. prodigiosus*, and *B. fluorescens*. Studying the lipase of *B. tuberculosis* and other bacteria, Wells and Corper (4), by testing the killed bacterial substance on esters and fats, demonstrated the presence of lipolytic enzymes in organisms which by the plate method apparently possessed no visible fat-splitting power. They further showed that sterile unheated emulsions of *B. tuberculosis*, while not actively lipolytic, possess enzymes capable of slowly hydrolyzing esters. Kendall, Walker, and Day (5) have demonstrated the occurrence of a soluble lipase in broth cultures of a variety of acid-fast organisms including tubercle bacilli of the human, bovine, and avian types. These authors found that the organisms, during the period of active growth, excrete a soluble lipase which occurs free in the medium.

Kendall and Simonds (6) have shown that sterile filtrates of plain and dextrose broth cultures of typhoid bacilli contain an esterase, capable of liberating acid from ethyl butyrate. The bacteria separated from the filtrates, however, showed but little esterase activity.

EXPERIMENTAL.

Methods.

Kastle and Loevenhart (7) have shown the advantage of using the lower esters, tributyrin and ethyl butyrate, in studying lipase activity. A preliminary experiment showed that when pneumococci are dissolved in bile the resultant solution contains an enzyme that splits both these esters. In the experiments to be recorded tributyrin has been used throughout as the substrate in the study of the intracellular lipase.

In determining lipase activity it has been customary to adjust the fat or ester substrate to approximate neutrality, with phenolphthalein as indicator, and then by titration to determine the amount of acid yielded by enzyme action. In the present study, however, it seemed more important to establish the optimum hydrogen ion concentration for action of the lipase, and then to maintain this reaction by the use of suitable buffer solutions. This buffered substrate maintains optimum conditions for enzyme action with a minimum of inhibition due to the acid products of hydrolysis. The amount of acid split off from the ester is determined by the amount of alkali required to readjust the digestion mixture to the initial reaction. It may also be calculated as the amount of acid required to change the buffered digestion mixture from the initial to the final hydrogen ion concentration. In the following experiments the ester was emulsified in 0.1 M phosphate solution of desired pH.

The method of preparing the enzyme solution, by dissolving the bacterial cells in sterile bile, was the same as that recorded in the experiments on the proteolytic enzymes of pneumococcus.

In no instances were antiseptics used as preservatives in the digestion mixtures. Sterility of all enzyme-substrate emulsions was proved by subculture.

Presence of an Intracellular Lipase and the Influence of Hydrogen Ion Concentration on Its Activity.

Experiment 1. (*a*) *Preparation of Enzyme.*—The bacterial residue from 4 liters of 18 hour plain broth culture of Pneumococcus Type II (No. F 208) was washed in isotonic salt solution, taken up in 20 cc. of sterile, undiluted ox bile, and held over night in the ice box. A portion of this enzyme solution was inactivated by heat.

(*b*) *Preparation of Substrate.*—2 per cent tributyrin (Kahlbaum) was emulsified in 0.1 M phosphate solution covering the range of pH values 4.9 to 7.8.

In carrying out the experiment 0.2 cc. of tributyrin was added to 10 cc. of sterile phosphate solution at the indicated reaction, and the mixtures were shaken until a fine emulsion was obtained. Three tubes were prepared at each reaction. 1 cc. of active enzyme solution was added to the first tube; 1 cc. of the same solution inactivated by heat to the second; and to the third 1 cc. of the undiluted bile used in preparing the bacterial solution. The tubes were then placed at 37°C. for 72 hours. The results of these experiments are given in Table I and are represented graphically in Text-fig. 1.

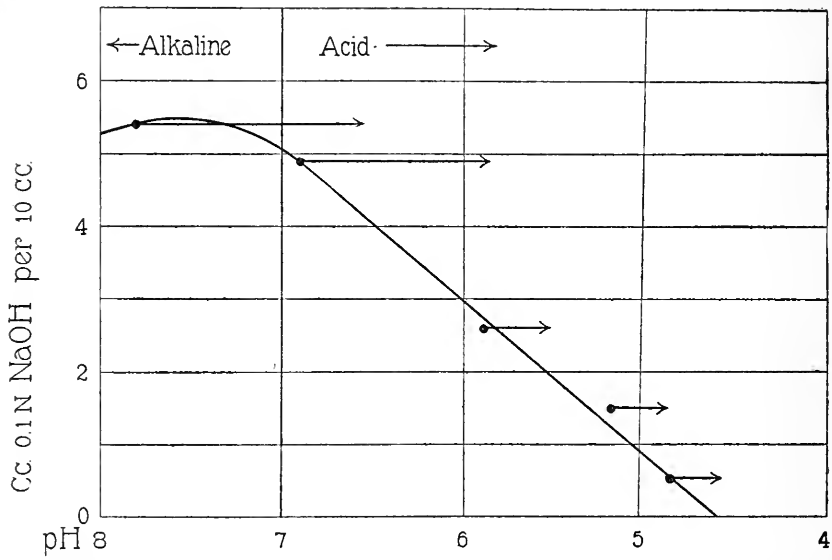
TABLE I.

Influence of Hydrogen Ion Concentration on the Activity of the Intracellular Lipase of Pneumococcus.

Initial reaction of digestion mixture.	Hydrogen ion concentration after 72 hrs. at 37°C. 10 cc. of 2 per cent tributyrin + 1 cc. of			10 cc. of mixture containing active enzyme readjusted with 0.1 N sodium hydroxide.	
	Bile.	Inactive enzyme.	Active enzyme.	To initial pH.	Amount required.
pH	*pH*	*pH*	*pH*	*pH*	*cc.*
4.9	4.9	4.9	4.6	4.9	0.3
5.2	5.2	5.2	4.9	5.2	1.5
5.9	5.9	5.9	5.5	5.9	2.3
6.9	6.9	6.9	5.8	6.9	4.9
7.8	7.8	7.8	6.5	7.8	5.4

The facts brought out in Experiment 1 demonstrate that within the pneumococcus cell there exists a markedly active lipase, or esterase. The acid formed from 10 cc. of 2 per cent tributyrin was equivalent to 5.4 cc. of 0.1 N alkali, or a normality of about 0.05 N butyric acid. The maximum activity of this esterase occurs at a reaction of about pH 7.8 and progressively decreases with increase in acidity. This

optimum reaction corresponds closely with that of the intracellular peptonase, and coincides with the optimum hydrogen ion concentration for growth of pneumococcus.



TEXT-FIG. 1. Influence of hydrogen ion concentration on the activity of the intracellular lipase of pneumococcus. The arrows indicate the extent of the reaction change.

Intracellular Nature of the Pneumococcus Lipase.

In the preceding paper it was shown that in filtrates of broth cultures of pneumococcus proteolytic enzymes were demonstrable only in the later phases of growth. Their appearance free in the culture medium coincided with the dissolution of the bacterial cells. In the early stages of growth, however, when cell multiplication is occurring at a maximum rate no enzymes are demonstrable in the culture filtrate. These facts indicate the intracellular nature of the proteolytic enzyme. Similarly it is shown in the following experiment that during the early phases of growth no lipase is present in cell-free filtrates. That the organisms were actively growing is evidenced from the change in reaction of the culture from pH 7.8 to 7.4.

Experiment 2.—20 cc. of the Berkefeld filtrate of a 5 hour culture of No. F 208 (the same preparation that was used in Experiment 8 in the preceding paper) were divided into two 10 cc. portions, one of which was autoclaved; 0.1 cc. of tributyrin was then added to each and the tubes were incubated for 48 hours. The results are given in Table II.

TABLE II.

Absence of Lipase in Culture Filtrates of Pneumococcus during the Period of Active Growth.

Filtrates from a 5 hour broth culture of Pneumococcus Type II.

Sterile filtrate.	Tributyrin.	Hydrogen ion concentration of filtrate.	
		Before incubation.	After incubation.
cc.	*cc.*	*pH*	*pH*
10 unheated.	0.1	7.4	7.4
10, heated.	0.1	7.3	7.3

From Table II it is evident that the lipase, like the peptonase, is an intracellular substance liberated on disintegration of the bacterial cell.

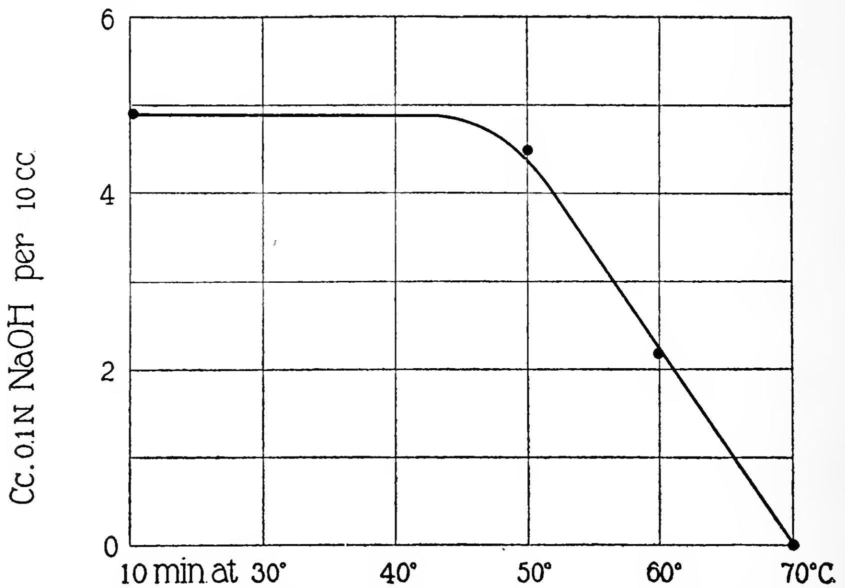
Effect of Heat on the Intracellular Lipase of Pneumococcus.

Experiment 3.—The thermostability of an enzyme in solution is influenced by the reaction of the medium in which it is dissolved and the length of exposure to the unfavorable temperature. In the present instance the enzyme was dissolved in bile, the solution adjusted to the optimum reaction for enzyme activity, pH 7.8, and subjected for 10 minutes in a water bath to the temperatures indicated.

The enzyme solution was the same as that used in Experiment 1 and was prepared from Pneumococcus Type II (No. F 208). 1 cc. portions of dissolved enzyme were carefully placed in sterile tubes and completely immersed in a water bath at the given temperature for 10 minutes. The tubes were immediately cooled to room temperature and to each were added 10 cc. of 2 per cent of tributyrin in 0.1 M phosphate solution, pH 7.8. After 24 hours at 37°C. the amount of acid split off from the ester by enzyme action was determined as in Experiment 1. The results are graphically presented in Text-fig. 2.

Variations in heat susceptibility of bacterial enzymes have been observed by many investigators. Söhngen (8), in studying the process of fat-splitting by bacteria, describes a lipase which resists a tempera-

ture of 100°C. for 5 minutes. The thermostability of the lipase of acid-fast bacteria has been noted by Wells and Corper, and by Kendall, Walker, and Day. These authors point out that heating to 100°C. for 15 minutes had little effect on the activity of the enzyme. Resistance of lipases in general to high temperatures is unusual; the various plant and tissue lipases in solution are as a rule inactivated by temperatures of 60–70°C. From Text-fig. 2 which illustrates the



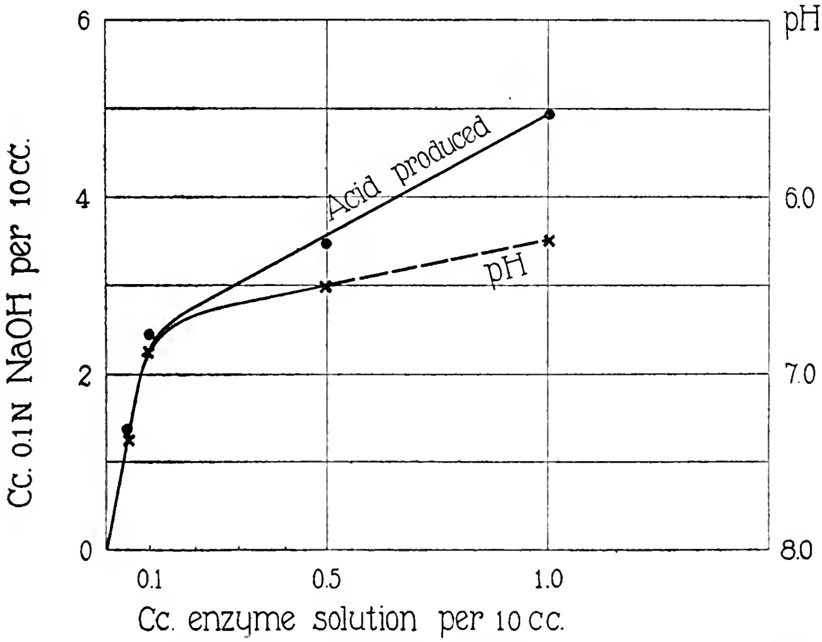
TEXT-FIG. 2. Heat stability of the intracellular lipase of pneumococcus.

effect on the pneumococcus lipase of exposure to various temperatures, it is evident that the dissolved enzyme suffers progressive loss of activity at temperatures above 50°C. and complete destruction at 70°C. for 10 minutes. According to Sternberg (9) the thermal death-point of pneumococcus is 52°C. for 10 minutes. The thermostability of the intracellular lipase under the conditions of this experiment, therefore, is somewhat greater than the heat resistance of the living organism.

Effect of Concentration of Enzyme on the Activity of the Intracellular Lipase of Pneumococcus.

If the ester-splitting property of bile solutions of pneumococci is enzymotic in nature, the rate of acid production should be proportional to the concentration of enzyme present. That this is the case is shown in the following experiment.

Experiment 4.—The enzyme solution, the same preparation used in the preceding experiment, was diluted with bile so that 1 cc. contained from 0.05 to 1 cc. of the original enzyme solution. These graduated quantities were added to 10 cc. of substrate consisting of 2 per cent tributyrin emulsified in 0.1 M phosphate solution of pH 7.8. The enzyme-substrate mixtures were incubated at 37°C. for 24 hours and the amount of acid hydrolysis was determined by the method outlined. The results are given in Text-fig. 3.



TEXT-FIG. 3. Influence of concentration of enzyme on the activity of the intracellular lipase of pneumococcus.

It is evident from the curves presented, that the rate of acid production by the action of the intracellular lipase on tributyrin is

directly proportional to the concentration of enzyme. Furthermore, as the enzyme concentration approaches the maximum, the amount of acid liberated by ester cleavage becomes so great that the resulting acidity is of itself sufficient to retard further enzyme action.

Relation of Virulence of Pneumococcus to Activity of the Intracellular Lipase.

Experiment 5. Preparation of Enzyme.—Two subcultures of the same strain of Pneumococcus Type II (No. F 208) were used. One of these, designated No. F 208 "B," the virulence of which had been greatly attenuated by cultural methods, failed to kill white mice in doses of 1 cc. of broth culture. The other, No. F 208 A, representing the original strain, the virulence of which had been preserved, was invariably fatal to these animals in doses of 0.000001 cc. injected intraperitoneally. The washed bacterial residues from 1,500 cc. of 15 hour plain broth cultures of these two organisms were collected and each was dissolved in 15 cc. of undiluted, sterile ox bile. The respective solutions were held over night in the ice box to ensure complete plasmolysis and were then tested for sterility on blood agar and in broth. The control enzyme solutions were inactivated by heat.

1 cc. portions of the active and inactivated enzyme preparations were added to tubes containing 10 cc. of 2 per cent tributyrin emulsified in sterile 0.1 M phosphate solution at pH 7.8. The digestion mixtures were then incubated for 24 hours at 37°C. Determinations of the hydrogen ion concentrations before and after incubation and of the amounts of acid produced in each instance are given in Table III.

TABLE III.

Relation of Virulence of Pneumococcus to Activity of the Intracellular Lipase.

Pneumococcus Type II.		Enzyme.	Hydrogen ion concentration.		Amount of 0.1 N sodium hydroxide per 10 cc. required to readjust to initial pH	Increase due to enzyme action.
Strain.	Minimum lethal dose for mice.		Initial.	Final.		
	cc.		*pH*	*pH*	*cc.*	*cc.*
F 208 "B"	Greater than 1	Active.	7.8	6.7	3.95	3.60
		Inactive.	7.8	7.7	0.35	
F 208 A	0.000001	Active.	7.8	6.7	3.75	3.45
		Inactive.	7.8	7.7	0.30	

Under the conditions of Experiment 5, in which were compared the relative potencies of the endoenzymes of an avirulent and of a virulent culture of the same strain of pneumococcus, loss of virulence was not associated with loss of enzyme activity.

Effect of Exposure to Acid Reaction on the Subsequent Activity of Pneumococcus Lipase.

Lord and Nye (10) have shown that pneumococci are rapidly killed at a hydrogen ion concentration of about pH 5.1. This acid death-point has been found both by these observers and by the present authors to correspond to the final reaction of broth cultures of pneumococcus when grown in the presence of sufficient carbohydrate. To determine whether this reaction is fatal to both organism and enzyme alike and whether this correlation has any significance in the mechanism of bile solubility the following experiment was performed.

Experiment 6.—1 cc. of active enzyme solution prepared as in Experiment 1 was added to 9 cc. of 0.1 M acid potassium phosphate solution of pH 4.6 (resulting reaction pH 5) and incubated at 37°C. for 2 hours. Then 0.83 cc. of N sodium hydroxide (calculated and verified on separate 9 cc. samples) was added to bring the acid enzyme solution to pH 7.8. After this readjustment of reaction 0.2 cc. of tributyrin was added as substrate, and the enzyme-substrate mixture incubated for 42 hours at 37°C. To serve as a control on activity, 1 cc. of the same enzyme solution, untreated, was added directly to 10 cc. of 0.1 M phosphate solution at pH 7.8 containing 2 per cent tributyrin (Table IV).

TABLE IV.

Effect on Lipase of Exposure to Acid Reaction.

Pneumococcus Type II.	Hydrogen ion concentration.		Amount of 0.1 N sodium hydroxide per 10 cc. required to readjust to initial pH.
	Initial.	Final.	
	pH	*pH*	*cc.*
Untreated enzyme	7.8	6.3	4.93
Acid treated and readjusted	7.8	6.4	3.80

From Table IV it is evident that after neutralization the activity of the pneumococcus lipase is little influenced by previous exposure

to a reaction of pH 5. The bile insolubility of pneumococci after similar acid treatment is apparently not attributable to the death of the lipase, but is possibly referable to changes in the cell protoplasm.

Effect of Age on the Activity of the Intracellular Lipase of Pneumococcus.

Experiment 7.—Enzyme solutions prepared from pneumococci by extraction in phosphate solution as described in the preceding paper possessed lipase activity entirely comparable to that of enzyme solutions prepared by the bile method. These preparations were still active after preservation for 7 weeks, as is shown in Table V.

TABLE V.

Age Stability of Lipase.

Pneumococcus Type II.	Age.	Hydrogen ion concentration.		Amount of 0.1 N sodium hydroxide per 10 cc. required to readjust to initial pH.
		Initial (0.1 M phosphate).	Final.	
	wks.	*pH*	*pH*	*cc.*
No. F 208	7	7.8	7.1	4.9
" II	3	7.8	6.9	6.5

DISCUSSION.

When pneumococci are dissolved in bile or extracted by the methods described, the resultant solution possesses, in addition to the proteolytic activity recorded in the preceding paper, a lipase (esterase) as measured by its power to split off acid from tributyrin.

Since a number of investigators (Hewlett (11), Magnus (12), and Loevenhart and Souder (13)) have shown that bile and bile salts not only do not interfere with lipase activity, but on the contrary accelerate the reaction, it was to be expected that the use of bile in effecting solutions of pneumococci would serve as an ideal method for demonstrating lipase activity. Bile, however, is not essential to the reaction, since extracts prepared by other methods exhibit comparable activity.

This esterase manifests its maximum activity at a hydrogen ion concentration of about pH 7.8, which is the optimum reaction for initiating growth of pneumococcus. The lipolytic activity of this enzyme progressively diminishes with increasing acidity until at about

pH 5 further hydrolysis ceases. That the point of acid extinction of esterase activity corresponds closely with the acid death-point of the living pneumococcus reveals another interesting correlation between cellular function and enzyme action. The rate of acid liberation from tributyrin during the initial stage of the action of the intracellular lipase is directly proportional to the concentration of enzyme. In the later phases of the reaction, the acid liberated by ester cleavage is sufficient in itself to retard further hydrolysis.

That the pneumococcus lipase is intracellular in nature is evidenced by the fact that it is present in maximum amount in bile solutions of washed bacterial cells, but cannot be demonstrated in culture filtrates during the period of active growth of the organism. The thermostability of the endolipase in solution is greater than the heat resistance of the living pneumococcus. After 10 minutes exposure in a water bath at temperatures greater than 50°C., the dissolved enzyme suffers progressive loss in activity until at 70°C. complete destruction results. Enzyme solutions preserved at refrigerator temperature retain their activity for weeks.

As to the possible relation of the activity of endoenzymes of pneumococcus to virulence of the living cell, the observations recorded in these studies are too limited to warrant discussion. Comparison of the relative potency of the endoenzymes of avirulent and virulent cultures of the same strain of pneumococcus has shown, however, that under the experimental conditions defined, loss of virulence was not associated with loss of enzymic activity.

Pneumococci exposed to a reaction corresponding to the acid death-point of the cell, that is, an acidity equivalent to or greater than pH 5, are thereby rendered insoluble in bile. This bile insolubility of pneumococci after acid treatment persists upon neutralization of the acid and even after the cells have been removed, washed, and resuspended in a neutral medium. In view of the possibility that the mechanism of bile solubility might in some way be related to the endolipase, in which instance the bile salts might function as coenzyme, or activator, it seemed pertinent to determine the effect of exposure to acid reaction on the subsequent activity of the enzyme itself. Since it has been shown, however, that upon neutralization the activity of the lipase is little influenced by previous exposure to a reaction of

pH 5, an acidity which kills the living cell, the phenomenon of bile insolubility of pneumococci after similar acid treatment is apparently not attributable to destruction of the endoenzyme.

SUMMARY.

1. Pneumococci contain an intracellular enzyme of marked lipolytic activity as measured by the acid liberated by its action on tributyrin.

2. Enzyme-containing solutions may be prepared by dissolving pneumococci in bile, or by extraction by other means.

3. The optimum reaction for maximum activity of the endolipase is about pH 7.8, which coincides with the optimum hydrogen ion concentration for growth of pneumococci.

4. Heating the enzyme for 10 minutes at 70°C. destroys its activity.

5. Attenuation of virulence of pneumococcus had no measureable effect on enzyme activity.

6. The possible relation of the endolipase to the mechanism of bile solubility is discussed.

BIBLIOGRAPHY.

1. Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1920, xxxii, 547.
2. Fuhrmann, F., Vorlesungen über Bakterienenzyme, Jena, 1907.
3. Eijkman, C., *Centr. Bakt.*, 1901, xxix, 841.
4. Wells, H. G., and Corper, H. J., *J. Infect. Dis.*, 1912, xi, 388.
5. Kendall, A. I., Walker, A. W., and Day, A. A., *J. Infect. Dis.*, 1914, xv, 443.
6. Kendall, A. I., and Simonds, J. P., *J. Infect. Dis.*, 1914, xv, 354.
7. Kastle, J. H., and Loevenhart, A. S., *Am. Chem. J.*, 1900, xxiv, 491.
8. Söhngen, N. L., *Koninkl. Akad. Wetensch., Wis- en Natuurk. Afd.*, 1911, xx, 126; abstracted in *Jahrb. Fortschr. Tierchem.*, 1911, xli, 788.
9. Sternberg, G. M., A text-book of bacteriology, New York, 2nd edition, 1901.
10. Lord, F. T., and Nye, R. N., *J. Exp. Med.*, 1919, xxx, 389.
11. Hewlett, A. W., *Bull. Johns Hopkins Hosp.*, 1905, xvi, 20.
12. Magnus, R., *Z. physiol. Chem.*, 1906, xlviii, 376.
13. Loevenhart, A. S., and Souder, C. G., *J. Biol. Chem.*, 1906–07, ii, 415.

STUDIES ON THE ENZYMES OF PNEUMOCOCCUS.

III. Carbohydrate-Splitting Enzymes: Invertase, Amylase, and Inulase.

By O. T. AVERY, M.D., and GLENN E. CULLEN, Ph.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 11, 1920.)

The two preceding papers (1, 2) on the enzymes of pneumococcus have dealt with the proteolytic and lipolytic activities of extracts of the bacterial cells, and of sterile filtrates of cultures. It has been pointed out that enzymes capable of actively hydrolyzing various substrates exist preformed within the cell and that by suitable methods they can be obtained free in solution and their action studied independently of the living organism.

The avidity with which pneumococcus attacks certain carbohydrates is manifest in the accelerated growth and increased acid production of organisms cultivated in the presence of these substances. Acids are produced in culture media from starches and glucosides, as well as from mono- and disaccharides. It has been customary to assume that these fermentation reactions are the summation of the action of several enzymes, first, the hydrolysis of the disaccharide or starch to monosaccharide, and secondly, the production of acid from the monosaccharide.

It was of interest, therefore, to determine whether the bile solutions of pneumococci which were known to contain protein- and fat-splitting enzymes also contained either or both groups of carbohydrate enzymes. Tests upon glucose, saccharose, starch, and inulin substrates were carried out with enzyme solutions containing peptonase and lipase prepared by dissolving pneumococci in bile.

The simple expedient of dissolving the bacterial cells in bile and testing the resultant solution for the presence of enzymes, a method admirably adapted to the determination of proteolytic and lipolytic activity, was found unsatisfactory for studying the enzymes attacking

carbohydrates, since bile in the concentration necessary to effect bacterial solution completely inhibits the activity of the enzymes converting sugar and starch. Consequently the method described below was adopted, by which disintegration of the pneumococcal cells is effected by suspending them in balanced phosphate solution at pH 6.2 and hastening physical disruption by repeated freezing and thawing of the bacterial suspension. The enzymes liberated from the bacterial bodies in this manner, as will be shown in the following experiments, are capable of hydrolyzing sucrose, starch, and inulin, the three test substances chosen for the typical reactions of invertase, amylase, and inulase.

EXPERIMENTAL.

Bacteriological Methods.

Preparation of Enzyme Solution by the Acetone Method.—Because of the fact that precipitation with acetone has been a satisfactory method for preparing and purifying many types of enzymes, an attempt was made to obtain an active carbohydrate-splitting enzyme from pneumococcus by the following procedure.

The residue of 2 liters of an 18 hour broth culture of pneumococcus was taken up in two 10 cc. portions of sterile distilled water, and each portion was poured into 150 cc. of acetone. After standing over night the supernatant solutions were removed from the precipitates which were then allowed to dry. Cultural examination showed that the organisms had been killed by acetone precipitation, although they remained intact and were Gram-positive.

One portion of the dry residue was shaken up with 10 cc. of 0.1 M phosphate at pH 7.4; the other was treated with 2 cc. of 0.1 N sodium hydroxide, allowed to stand for several hours, then neutralized with 0.1 N hydrochloric acid to pH 7.4, and diluted to 10 cc. Each portion was tested for lipase, for peptonase, and for invertase. The tests were all negative.

Preparation of Enzyme Solution at pH 6.2.—Previous work has shown that growth of pneumococcus cannot be initiated at a reaction more acid than pH 6.8 (3), and that disintegration of the bacterial

cell occurs most rapidly at about pH 6.2. Moreover, although the subsequent activity of the intracellular lipase and peptonase at optimum reactions is not materially influenced by temporary exposure to an acidity as great as pH 5, they show much less activity at pH 6.2 than at a neutral or slightly alkaline reaction. It seemed probable therefore, that disintegration of the pneumococcus cell, under conditions at which the autolytic processes are at a minimum and at which neither initial growth of the organism nor destruction of known enzymes occurs, might liberate the intracellular carbohydrate-splitting enzymes. Such conditions were obtained in the following manner.

The washed bacterial residue from 1.5 liters of an 18 hour plain broth culture of pneumococcus was taken up in 15 cc. of 0.1 M phosphate solution of pH 6.2, and placed in the ice box until intact bacterial cells could no longer be found under the microscope. After the solution had been proved sterile, it was tested for the presence of known enzymes. The lipase activity of this solution was comparable to that obtained with the bile solution previously used.

Preparation of Enzyme Solution by Cytolysis with Alternate Freezing and Thawing.—This method differed from the preceding only in that the disintegration of the cell was hastened by repeated freezing and thawing. An ice-salt mixture of about $-22°C$. was used.

Sterility Controls.—In addition to the sterility tests on the enzyme solution, the final enzyme-substrate mixtures were tested by transferring 0.1 cc. to 5 cc. of broth and incubating for 48 hours.

Chemical Methods.

Preparation of Substrates. Sucrose.—A 4 per cent sucrose solution was sterilized in boiling water for 20 minutes. 25 cc. of this sterile 4 per cent sugar solution were then added to 25 cc. of sterile phosphate solution of the desired pH.

Glucose.—Prepared in the same manner as sucrose.

Starch.—A 2 per cent and a 0.2 per cent suspension of Kahlbaum's rice starch in 0.1 M phosphate solution of pH 7.4 were autoclaved for 20 minutes at 15 pounds pressure.

Inulin.—A 2 per cent inulin solution in 0.1 M phosphate at pH 7.4 was sterilized as above.

Phosphate Solutions.—The 0.1 M phosphate solutions for the range pH 5 to 8.3 were prepared from potassium acid phosphate and sodium phosphate (Na_2HPO_4) according to Sörensen's tables. For the more acid range, mixtures of 0.1 M potassium acid phosphate and 0.1 N hydrochloric acid were used. In all the experiments the appropriate corrections were made for the effect on the reaction of the enzyme solution. All phosphate solutions were sterilized by autoclaving for 20 minutes at 15 pounds pressure.

Hydrogen Ion Concentration.—The hydrogen ion concentration was ordinarily determined by the colorimetric method. For the range of acidity greater than pH 4.5 and for frequent controls of the colorimetric standards, the electrometric method, using Clark's rocking electrodes (4), was employed.

Determination of Reducing Sugar.—Qualitative tests for reducing sugar were carried out by using 5 cc. of Benedict's qualitative solution and 0.5 cc. of test solution and boiling for 10 minutes.

Quantitative sugar determinations were made with either Benedict's quantitative titrating method, by determining the rotation of the solution, or by the gravimetric copper method.

Carbon Dioxide.—Determinations were made with Van Slyke's apparatus (5).

Amylase Action.—Hydrolysis of starch to dextrins was determined by means of the iodine color test. 1 cc. of the solution was diluted with 2 cc. of water and 0.2 cc. of a dilute iodine solution (about $\frac{N}{150}$) added. Hydrolysis of the starch or dextrin to reducing sugar was determined as indicated above.

Action of Intracellular Enzymes of Pneumococcus on Carbohydrates.

Experiment 1.—A solution of Pneumococcus Type II prepared as outlined above was added to a series of tubes containing saccharose, inulin, starch, glucose, and glucose-peptone mixture. The glucose-peptone solution was used with the idea that the nitrogen of the peptone might accelerate glucose hydrolysis. The tubes were incubated for 48 hours at 37°C. The solutions were then analyzed as described. The results are given in Table I.

Experiment 2.—This experiment differed from the preceding one in that the suspension of pneumococci was frozen and thawed five times. The enzyme solution was held at ice box temperature (4°C.) for 16 days until culture controls in blood broth no longer showed the presence of viable organisms.

In addition to the substrates used in the preceding experiment two additional control tubes were included, one containing 10 cc. of a 2 per cent saccharose solution in 0.1 M phosphate solution plus 1 cc. of bile, and the second containing 0.2 per cent glucose. Since slight glucose fermentation might have been masked by an excess of the sugar, the dilute solution was included in this series. The results are presented in Table II.

TABLE I.

Action of Intracellular Enzymes of Pneumococcus on Carbohydrates.

Enzyme solution prepared by cytolysis at pH 6.2.

Substrate in 0.1 M phosphate solution.	Final hydrogen ion concentration.		Qualitative determinations with Benedict's solution.		Color with iodine.		Rotation.		Carbon dioxide content per 2 cc.	
	Inactive.	Active.	Inactive.	Active.	Inactive.	Active.	Inactive.	Active.	Inactive.	Active.
	pH	*pH*							*cc*	*cc.*
Saccharose, 2 per cent.	7.4	7.4	–	++++			2.7°	1.36°	0.075	0.080
Glucose, 2 per cent.	7.4	7.4	++++	++++					0.075	0.075
Glucose, 1 per cent, in 1 per cent peptone.	7.4	7.4	++++	++++					0.075	0.090
Inulin, 2 per cent.	7.4	7.4	–	++						
Starch, 2 per cent.	7.4	7.4	–	+	Blue.	Lavender.	0°	0.2°		
Starch, 0.2 per cent.	7.4	7.4	–	+	"	Vanishing light lavender.				
Tributyrin,* 1 per cent.	7.8	6.3								

* Tributyrin was used as a control of enzyme activity.

It is evident from Tables I and II that pneumococcus contains enzymes that hydrolyze starch to dextrins (amylase), hydrolyze inulin (inulase), and invert saccharose to reducing sugars (invertase). The microorganism appears, therefore, to contain enzymes capable of hydrolyzing complex carbohydrates into simple sugars. Bile

completely inhibits the hydrolysis; whether this is due to destruction of the enzyme or to inhibition of its action has not been determined. This fact explains the failure to detect the carbohydrate enzymes in the bile solution used for the study of the peptonase and lipase of pneumococcus. On the other hand, the peptonase and lipase are as active in the solution obtained by the method described as in the bile solutions.

All attempts to demonstrate an enzyme capable of fermenting glucose or producing acid from glucose were unsuccessful.

TABLE II.

Action of Intracellular Enzymes of Pneumococcus on Carbohydrates.

Enzyme solution prepared by alternate freezing and thawing.

Substrate in 0.1 M phosphate solution.	Hydrogen ion concentration.		Qualitative determinations with Benedict's solution.	Color with iodine.	Carbon dioxide content per 2 cc.
	Initial.	Final.			
	pH	*pH*			*cc.*
Glucose, 2 per cent	7.3	7.3	++++		0.09
" 0.2 per cent	7.3	7.3	+++		
Starch, 2 per cent	7.3	7.3	++	Lavender.	
" 0.2 per cent	7.3	7.3	+	Vanishing lavender.	0.07
" 0.2 " " (control)	7.3	7.3	−	Blue.	
Inulin, 2 per cent	7.3	7.3	+		
Saccharose, 2 per cent	7.3	7.3	++++		0.07
" + 1 cc. of bile	7.3	7.3	−		
Control	7.3	7.3	−		
Tributyrin,* 1 per cent	7.8	6.3			

* To control enzyme activity.

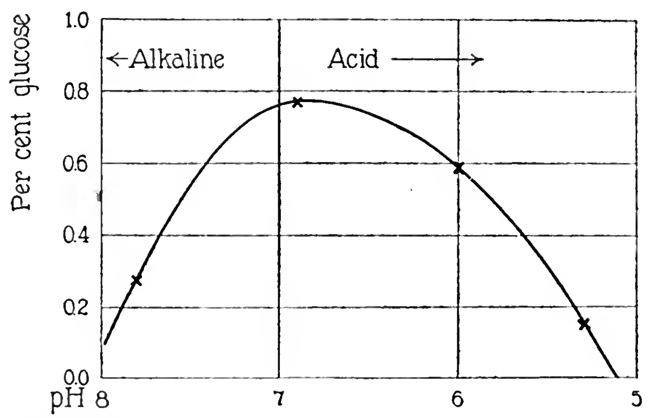
Intracellular Nature of the Carbohydrate-Splitting Enzymes.

Experiment 3.—Tests for invertase, amylase, and for a glucose-fermenting enzyme in the filtrate from a young actively growing culture of pneumococcus were carried out exactly as in the case of lipase and peptonase. Saccharose, starch, and glucose to make 1 per cent solutions were added to the sterile filtrate from a 5 hour culture of Pneumococcus Type II, and the mixture was incubated for 48 hours at 37°C. The mixtures were tested as in the preceding experiment and no evidence of enzyme action was demonstrable. Since in the solutions of pneumococci

obtained by the method described there are active carbohydrate-splitting enzymes, their absence in the filtrates of cultures during the early phase of growth, before cell disintegration has occurred, indicates that, like lipase and peptonase, these enzymes are intracellular in nature.

Influence of Hydrogen Ion Concentration on the Activity of the Intracellular Invertase of Pneumococcus.

The preceding experiments have demonstrated the presence of several carbohydrate-splitting enzymes. It now seemed desirable to determine the influence of the hydrogen ion concentration on their activity.



TEXT-FIG. 1. Influence of hydrogen ion concentration on the activity of the intracellular invertase of pneumococcus.

Experiment 4. (*a*) *Preparation of Enzyme Solution.*—The solution was prepared as described in preceding experiments.

(*b*) *Preparation of Substrate.*—The substrates were prepared as outlined under Methods. 1 cc. of enzyme was added to 10 cc. of substrate solution; a duplicate tube without enzyme served as control for acid hydrolysis.

Both qualitative and quantitative reducing sugar determinations were made. The results are given in Table III and Text-fig. 1.

Optimum Hydrogen Ion Concentration for Amylase Action.

These experiments were carried out as in the case of invertase. Although quantitative results were not carried out, the iodine color indicates that the optimum reaction is about pH 7.

TABLE III.

Influence of Reaction on Invertase Action.

Enzyme preparation.	Hydrogen ion concentration.	Glucose determinations.		
		Qualitative determinations with Benedict's solution.	Quantitative determinations by Benedict's method.	Quantitative determinations by the gravimetric copper method.
	pH		*per cent*	*per cent*
No. D 39	7.7	++	0.1	
" D 39	6.9	+++	0.51	
" D 39	6.1	+++	0.34	
" D 39	5.2	+	0.1	
" D 39	4.8	−	0.0	
" F 208	7.8	+±		0.29
" F 208	6.9	+++		0.77
" F 208	6.0	++		0.59
" F 208	5.3	+		0.15
" F 208	4.85	−		

All controls negative.

TABLE IV.

Influence of Reaction on Amylase Action.

Enzyme. preparation.	Hydrogen ion concentration.	Qualitative determinations with Benedict's solution.	Color with iodine.
	pH		
No. D 39	8.2	−	Deep, fading blue.
" D 39	6.9	++	Red-lavender.
" D 39	6.1	+	Blue-lavender.
" D 39	5.1	−	Blue.
" D 39	4.8	−	
" F 208	7.8	±	Very pale lavender, fading instantly.
" F 208	6.9	++	Pale lavender, fading rapidly.
" F 208	6.0	++	Deep " " slowly.
" F 208	5.3	−	Faint change only.
" F 208	4.8	−	Blue as controls.

It is evident from Tables III and IV that the optimum hydrogen ion concentration for pneumococcus invertase and amylase is about pH 7.

DISCUSSION.

It is generally accepted that in the utilization of carbohydrates by living bacteria, hydrolysis of these complex substances is brought about through the action of enzymes. The fact has been recognized that enzymes capable of converting sucrose and starch may be found in fungi, especially in yeasts and moulds. Studies on the carbohydrate-splitting processes of invertase-producing bacteria have also been reported by numerous investigators. The earlier work of Fermi and Montesano (3), particularly, lists a number of microorganisms, in sterile cultures of which invertase activity was demonstrable. The isolation and study of carbohydrate-splitting enzymes apart from the living cell have not, so far as we have been able to find in the literature, been attempted in the case of pneumococcus.

The demonstration of the intracellular agents concerned in carbohydrate cleavage by pneumococcus was accomplished by a method through which the release of endoenzymes from the intact organism was effected by breaking down of the cell structure under conditions not injurious to the reactive substances themselves. That physical disruption of the cell membrane through alternate freezing and thawing is subsequently followed by autolytic processes is indeed likely; that the chemical changes brought about by autolysis under these conditions, however, exert but slight influence on the activity of the enzymes studied is evidenced by the avidity with which hydrolysis occurs, and the length of time during which potency is preserved.

From the data presented in this and the two preceding papers, it may be concluded that within the cell bodies of pneumococci there exist in addition to the endohemotoxin described by Cole (6), a series of intracellular enzymes. The proteolytic and lipolytic functions of this endoenzyme-complex have already been described. In addition, there may now be added the activity of the endoenzymes causing hydrolysis of carbohydrate substances, such as sucrose, starch, and inulin.

The optimum hydrogen ion concentration for the invertase and amylase of pneumococcus is about pH 6.8 to 7. This represents a reaction slightly less alkaline than that shown to be optimum for the activity of the peptonase and esterase. The reaction most favorable

for the activity of enzymes that attack carbohydrates is not the same even for those having similar activities. Sörensen (7) has shown that the optimum hydrogen ion concentration for the invertase of beer yeast is about pH 4.5. In studying the influence of hydrogen ion concentration on the enzymic activity of three typical amylases of different origin, Sherman, Thomas, and Baldwin (8) found that the starch-splitting enzymes of malt and *Aspergillus oryzæ* are both most active at an acid reaction (pH 4.4 to 4.8), while the pancreatic amylase reaches its maximum at pH 7. The carbohydrate-splitting enzymes of pneumococcus function best at a neutral reaction and are operative over a zone which corresponds closely to the reaction range of the living organism when grown in the presence of fermentable substances (9). Attempts to determine the presence of an enzyme or enzymes capable of fermenting dextrose and producing acid, an action characteristic of the growing cell, have been unsuccessful.

The invertase, amylase, and inulase of pneumococcus, like the proteolytic and lipolytic enzymes, are intracellular in nature and are found free in culture fluids only after cell disintegration has begun.

SUMMARY.

1. A method is described for the preparation of an active enzyme-containing solution of pneumococci, in which no living cells are present. These enzymes are capable of hydrolyzing sucrose, starch, and inulin.

2. The invertase and amylase of pneumococcus are active within the limits pH 5 to 8, with an optimum reaction of about pH 7. This reaction range corresponds closely with limiting hydrogen ion concentrations which define growth of the organism in the presence of carbohydrate.

3. These studies indicate that the enzymes described are not true secretory products of the living cell, but are of the nature of endoenzymes, since their activity can be demonstrated only when cell disintegration has occurred.

BIBLIOGRAPHY.

1. Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1920, xxxii, 547.
2. Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1920, xxxii, 571.
3. Fermi, C., and Montesano, G., *Centr. Bakt., 2te Abt.*, 1895, i, 542.
4. Clark, W. M., *J. Biol. Chem.*, 1915, xxiii, 475.
5. Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.
6. Cole, R., *J. Exp. Med.*, 1914, xx, 346.
7. Sörensen, S. P. L., *Biochem. Z.*, 1909, xxi, 131.
8. Sherman, H. C., Thomas, A. W., and Baldwin, M. E., *J. Am. Chem. Soc.*, 1919, xli, 231.
9. Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1919, xxx, 359.

EPINEPHRINE CONTENT OF THE SUPRARENAL GLANDS IN ANAPHYLAXIS.

By MAURICE I. SMITH, M.D., AND S. RAVITZ, M.D.

(From the Pharmacological Laboratory of the University of Nebraska Medical College, Omaha.)

PLATE 89.

(Received for publication, June 5, 1920.)

Since the discovery of the pressor action of the internal secretion of the suprarenal glands by Oliver and Schäfer (1) many attempts have been made to assign to these glands a function of considerable importance, that of maintaining the normal tone of the vascular system in combating agencies which tend to lower the blood pressure and produce shock-like phenomena. The suprarenal glands were examined histologically in different conditions of shock with somewhat variable results. Bainbridge and Parkinson (2) found by staining methods that epinephrine entirely disappeared from the suprarenal glands in cases of death from acute infections, and in postoperative shock. Hornowski (3) likewise found, by staining methods, that the epinephrine disappears from the suprarenals in postoperative shock, and he believes the latter to be due to epinephrine depletion. Corbett (4) states that the epinephrine store of the suprarenals is depleted to the extent of from 75 to 3 per cent of the normal by various experimental procedures leading to shock. Cramer (5), on the other hand, using histochemical methods, found the suprarenals actually loaded with epinephrine granules, and actively secreting them into the central vein in cases of postoperative shock, at a time when the blood pressure was low and the temperature subnormal.

The epinephrine output of the suprarenals in various conditions of experimental shock has been investigated by Bedford (6), who found that the epinephrine content of adrenal blood was greatly augmented during shock, and this he believes to be associated with an increased activity of the glands, a mechanism to save the animal from shock and low blood pressure. Stewart and Rogoff (7), making quantitative estimations of the rate of output of epinephrine from the suprarenal glands in a given unit of time, found that this was not influenced by low blood pressure such as occurs after various procedures to induce shock.

The condition of the suprarenal glands in anaphylactic shock and during the state of sensitization does not seem to have been examined, either as regards pathological changes therein, or from the standpoint of their functional activity, although pathological changes in the liver, kidney, muscle, myocardium, spleen,

and blood vessels have been described in experimental acute and chronic anaphylaxis (Gay and Southard (8), Longcope (9), and Boughton (10)). In view of the alleged alterations of the epinephrine content and output of the suprarenal glands in various forms of surgical shock, it seemed probable that the suprarenal glands of animals in anaphylactic shock might show similar changes, especially so since the experiments of Hirschfeld and Hirschfeld (11) suggest the presence of a vasoconstricting substance in the blood of animals in anaphylactic shock. Furthermore, a more or less beneficial effect has been shown to be produced by the administration of epinephrine to animals in anaphylactic shock (Galambos (12)).

We therefore undertook to determine the epinephrine content of the suprarenal glands in animals sensitized to a foreign protein, as well as in those subjected to varying degrees of anaphylactic shock. Guinea pigs and rabbits were used in the experiments. The animals were sensitized by a subcutaneous or intraperitoneal injection of ox serum, and after an incubation period of at least 2 weeks they were reinjected with the antigen intravenously. Immediately after death or at the height of shock the suprarenal glands were removed,[1] weighed, and their epinephrine content was determined. We have also determined the epinephrine content of the suprarenals of sensitized, but not shocked animals. In the latter group the animals were killed at the height of sensitization by a blow on the neck. All the control animals were treated in the same manner.

The epinephrine estimations were made, for the most part, by the colorimetric method of Folin, Cannon, and Denis (13). We have not, however, used their uric acid standard, but have employed instead a freshly prepared solution of epinephrine hydrochloride made from the adrenalin of Parke, Davis and Company.

The results of the experiments are summarized in Table I, from which it will appear that the epinephrine content of the suprarenal glands in sensitized and in shocked animals does not materially differ from that of normal animals. The average epinephrine content of the suprarenals of normal guinea pigs was 0.073 mg. per 100 mg. of gland, that of sensitized guinea pigs 0.066 mg. per 100 mg. of gland, while that of guinea pigs dying in anaphylactic shock was 0.072 mg. per 100 mg. of gland. The epinephrine content of the suprarenals of normal rabbits averaged 0.30 mg. per 100 mg. of gland, that of a

[1] All operations were performed under ether anesthesia.

fully sensitized rabbit was found to be 0.27 mg. per 100 mg. of gland, while the average epinephrine content of rabbits in anaphylactic shock for periods varying from 2 minutes to 3 hours appeared to be 0.28 mg. per 100 mg. of gland.

TABLE I.

Epinephrine Content of the Suprarenals in Normal, Sensitized, and Shocked Animals Determined by the Colorimetric Method.

Experiment No.	Weight.	Condition.	Weight of suprarenals.	Epinephrine. Total.	Epinephrine. Amount per 100 mg. of gland.
		Guinea pigs.			
	gm.		*mg.*	*mg.*	*mg.*
1	410	Normal.	270	0.19	0.070
2	460	"	350	0.29	0.083
3	465	"	230	0.15	0.065
4	455	Sensitized.	290	0.18	0.062
5	468	"	270	0.19	0.070
6	385	Anaphylactic shock; died in 4 min.	160	0.13	0.081
7	450	" " " " 3 "	230	0.17	0.074
8	355	" " " " 4 "	180	0.09	0.050
9	455	" " " " 3 "	250	0.16	0.064
10	425	" " " " 4 "	280	0.20	0.072
11	378	" " " " 3 "	230	0.21	0.091
12	442	" " " " 4 "	220	0.16	0.073
13	363	" " " " 4 "	320	0.24	0.075
14	445	" " " " 4 "	330	0.22	0.067
		Rabbits.			
	kg.				
15	1.9	Normal.	320	1.27	0.39
16	1.9	"	150	0.33	0.22
17	2.0	Sensitized.	230	0.63	0.27
18	1.5	In anaphylactic shock 20 min.; killed.	100	0.37	0.37
19	1.5	Anaphylactic shock; died in 2 min.	100	0.25	0.25
20	1.7	" " " " 3 hrs.	280	0.63	0.23

The chemical method for estimation of epinephrine such as we employed in the above experiments may be open to objection, since substances other than epinephrine, notably uric acid, give similar color reactions. It was thought desirable, therefore, to confirm the

above findings by some other method, and a few experiments were made to determine the epinephrine content of the suprarenals of normal, sensitized, and shocked guinea pigs by the segment method of the surviving rabbit intestine after the method of Cannon and de la Paz (14) and Hoskins (15). The suprarenal glands were extracted with normal saline solution, and assayed for their epinephrine content against a freshly diluted solution of adrenalin hydrochloride 1:1,000 of Parke, Davis and Company. In some of the experiments (Nos. 26 to 29, Table II) the suprarenals were extracted, as for the

TABLE II.

Epinephrine Content of the Suprarenals in Normal, Sensitized, and Shocked Guinea Pigs Determined by the Biological Method.

Experiment No.	Weight.	Condition.	Weight of suprarenals.	Epinephrine. Total.	Epinephrine. Amount per 100 mg. of gland.
	gm.		*mg.*	*mg.*	*mg.*
21	400	Normal.	250	0.10	0.04
22	400	"	250	0.20	0.08
23	425	Sensitized.	270	0.10	0.04
24	425	"	300	0.12	0.04
25	404	In anaphylactic shock.	350	0.15	0.04
26	445	Anaphylactic shock; died in 4 min.	330	0.10	0.03
27	363	" " " " 4 "	320	0.10	0.03
28	442	" " " " 4 "	220	0.15	0.07
29	378	" " " " 4 "	230	0.08	0.03

colorimetric method, with 1 cc. of 0.1 N hydrochloric acid in 8.5 cc. of water, and heated to boiling. 0.5 cc. of 10 per cent sodium acetate was then added, and the whole filtered. The standard for these experiments was made in the same manner; *i.e.*, a solution was made to contain 1 mg. of epinephrine in 10 cc. containing 1 cc. of 0.1 N hydrochloric acid and 0.5 cc. of 10 per cent sodium acetate. This was further diluted with water, as required. The segment of rabbit intestine was always suspended in the same volume of Tyrode solution, oxygenated and kept at a constant temperature of 38°C. Fig. 1 illustrates the method.

The results of these experiments are presented in Table II. It will be seen that the epinephrine content of the suprarenals, as in the preceding experiments, runs practically the same in the sensitized and shocked animals as in the normal controls.

It is not desired to draw too far reaching conclusions from these experiments. They show clearly that the epinephrine store of the suprarenal glands is not materially altered by sensitization or by varying degrees of anaphylactic shock. Neither the epinephrine output nor the activity of the suprarenal medulla in sensitized and shocked animals, however, can be judged from these experiments, as Stewart, Rogoff, and Gibson (16) have pointed out that there is no necessary relation between the store of epinephrine in the suprarenals and their output; and Kuriyama (17) has also recently come to the conclusion that the secretory activity of the suprarenal medulla cannot be judged by its epinephrine content. Nevertheless, with the epinephrine content of the suprarenals remaining practically normal during sensitization and anaphylactic shock, it is highly improbable that there is any relation between these phenomena and the chromaffin substance of the suprarenals, such as has been claimed by some authors for surgical shock.

CONCLUSION.

The epinephrine store of the suprarenal glands remains unaltered in guinea pigs and rabbits which have been sensitized to ox serum, or subjected to varying degrees of anaphylactic shock by the reinjection of the foreign protein.

BIBLIOGRAPHY.

1. Oliver, G., and Schäfer, E. A., *J. Physiol.*, 1895, xviii, 230.
2. Bainbridge, F. A., and Parkinson, P. R., *Lancet*, 1907, i, 1296.
3. Hornowski, J., *Virchows Arch. path. Anat.*, 1909, cxcviii, 93.
4. Corbett, J. F., *J. Am. Med. Assn.*, 1915, lxv, 380.
5. Cramer, W., *Rep. Imperial Cancer Research Fund*, 1919, vi, 1.
6. Bedford, E. A., *Am. J. Physiol.*, 1917, xliii, 235.
7. Stewart, G. N., and Rogoff, J. M., *Am. J. Physiol.*, 1919, xlviii, 22.
8. Gay, F. P., and Southard, E. E., *J. Med. Research*, 1907, xvi, 143.
9. Longcope, W. T., *J. Exp. Med.*, 1913, xviii, 678; 1915, xxii, 793.
10. Boughton, T. H., *J. Immunol.*, 1916, i, 105; 1916–17, ii, 501; 1919, iv, 213.

11. Hirschfeld, H., and Hirschfeld, L., *Z. Immunitätsforsch., Orig.*, 1912, xiv, 466.
12. Galambos, A., *Z. Immunitätsforsch., Orig.*, 1913, xix, 437.
13. Folin, O., Cannon, W. B., and Denis, W., *J. Biol. Chem.*, 1912–13, xiii, 477.
14. Cannon, W. B., and de la Paz, D., *Am. J. Physiol.*, 1911, xxviii, 64.
15. Hoskins, R. G., *J. Pharmacol. and Exp. Therap.*, 1911–12, iii, 93.
16. Stewart, G. N., Rogoff, J. M., and Gibson, F. S., *J. Pharmacol. and Exp. Therap.*, 1916, viii, 205.
17. Kuriyama, S., *J. Biol. Chem.*, 1918, xxxiv, 299.

EXPLANATION OF PLATE 89.

Fig. 1. Epinephrine content of the suprarenal glands determined by the biological method. *B* was a solution of adrenalin chloride, 1 cc., containing 0.01 mg. *C* contained 0.001 mg. per cc. The suprarenals of Guinea Pig 115 (Experiment 26, Table II) were extracted as for the colorimetric method, and diluted 10 and 100 times respectively.

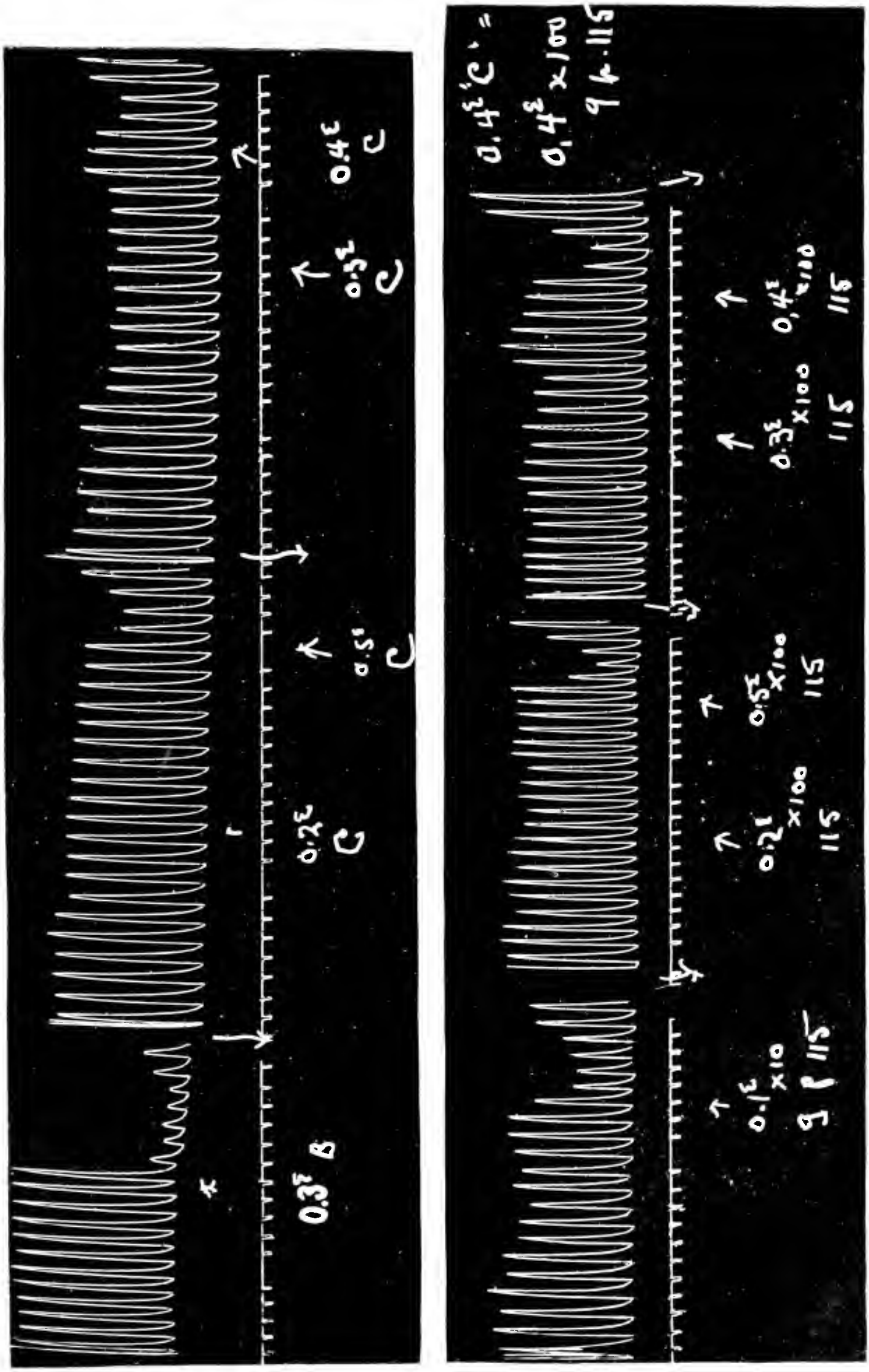


Fig. 1.

EXPERIMENTAL STUDIES ON YELLOW FEVER OCCURRING IN MERIDA, YUCATAN.

By HIDEYO NOGUCHI, M.D., and I. J. KLIGLER, Ph.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, April 3, 1920.)

INTRODUCTION.

In 1918 a leptospira was detected in the blood and liver from certain cases of yellow fever in Guayaquil.[1] The organism was demonstrated also in the blood and in organ emulsions from guinea pigs which had been inoculated with blood or liver emulsions from yellow fever patients and had shown typical symptoms of the disease, fever, hemorrhages, jaundice, and nephritis. A pure culture of the same organism was made directly from the blood of yellow fever cases and the blood of experimentally infected guinea pigs, and the pathogenicity of the culture was demonstrated in guinea pigs, marmosets, and pups. The organism, which has been designated *Leptospira icteroides*, was found to be a filter passer, easily destroyed by a temperature of 55°C. within 5 minutes, an obligatory aerobe, and requiring for growth a certain amount of unmodified blood serum from man or suitable animals. Its growth is usually suppressed by secondary bacterial contamination, but when successfully obtained it remains almost invisible to the naked eye, forming neither a discrete colony nor a striking change of the culture medium, and hence the culture may be overlooked under ordinary circumstances as sterile. An ordinary microscope does not render it visible in the fresh state, and staining with ordinary dyes fails to bring out its presence. Through a dark-field microscope with a powerful illumination, however, it can readily be recognized. In the blood of yellow fever patients the organism may be present in such small numbers that an average dark-field search usually misses it. From the comparatively small percentage of positive transmissions from yellow fever patients to guinea pigs, it seems probable that many strains of the organism fail to infect this animal fatally or even with moderate severity. Whenever a secondary bacterial infection occurs after an attempt at transmission, either the animal dies before the infection with *Leptospira icteroides* fully develops, or the course of infection is atypically modified and the strain thereby lost. The various factors just enumerated no doubt constitute some of the reasons why animal inoculation or the detection of the organism has not been successful in the hands of different investigators at an earlier period.

---

[1] Noguchi, H., *J. Exp. Med.*, 1919, xxix, 547, 565, 585.

It has been shown also[2] that the majority of the serums from persons recently recovered from an attack of yellow fever possess a specific dissolving power upon the organism when tested by the Pfeiffer reaction. This phenomenon has been regarded as significant in establishing a possible relation between yellow fever and *Leptospira icteroides*. Another important point with respect to the etiological relation was furnished by the experimental transmission of the leptospira infection from yellow fever patients to normal guinea pigs, or from infected guinea pigs to normal guinea pigs, by the bite of infected stegomyia females,[3] or with an emulsion of such mosquitoes, 8 days or longer after they had fed on infected individuals or animals.

By analogy *Leptospira icteroides* presented the principal characteristics of the virus of yellow fever as experimentally determined by Reed, Carroll, Agramonte, and Lazear[4] and by later investigators (Marchoux, Salimbeni, and Simond,[5] and Parker, Beyer, and Pothier[6]). Both are filter passers, readily killed at 55°C., transmitted by *Stegomyia calopus* after a period of incubation, not amenable to cultivation in ordinary media, invisible under the ordinary microscope, not resisting bacterial putrefaction, but capable of preservation in citrated blood under a layer of liquid paraffin for several days at room temperature,[5] inoculable by hypodermic inoculation (in the case of the yellow fever virus inoculation was made into volunteer human beings,[4, 5] in the case of *Leptospira icteroides* into susceptible animals), and producing similar symptoms. From these circumstances it seemed certain that *Leptospira icteroides* was responsible for the infection in the cases from which the organism was isolated or in which a positive Pfeiffer reaction was obtained, but the question as to whether or not *Leptospira icteroides* is also responsible for the disease known as yellow fever elsewhere than in Guayaquil was left open to further experimental determination.

The present paper gives the results obtained by us during a recent expedition to Merida, Mexico.[7] Merida, a city of 100,000 inhabitants, and the capital of Yucatan, has a small proportion of non-

[2] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 9.

[3] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 401.

[4] Reed, W., Carroll, J., Agramonte, A., and Lazear, J. W., *Senate Doc. No. 822, 61st Cong., 3rd Sess.*, 1911, 56.

[5] Marchoux, Salimbeni, and Simond, *Ann. Inst. Pasteur*, 1903, xvii, 665.

[6] Parker, H. B., Beyer, G. E., and Pothier, O. L., *Bull. Hyg. Lab., U. S. P. H., No. 13*, 1903.

[7] This expedition was undertaken under the auspices of the International Health Board of The Rockefeller Foundation and The Rockefeller Institute for Medical Research during the months of Dec., 1919, and Jan., 1920. The Commission received hearty and efficient cooperation from the hospital and sanitary officials in Merida, to whom we wish to express our appreciation and thanks.

immune foreign population, but it owes its previous epidemics of yellow fever to the occasional influx of foreigners or of troops sent thither from other and mountainous states where there is no yellow fever. It is reported that a systematic anti-stegomyia campaign had kept the city free from yellow fever during the 4 years preceding 1919, and that it was owing to an enormous crop of stegomyias in 1919 that an epidemic broke out in July and lasted until November, claiming about 100 patients among the newly arrived troops. The death rate was about 50 per cent.

At the time when one of us arrived in Merida,[8] there had been no new case of yellow fever for 13 days. On December 15 a soldier, J. M., was brought to the yellow fever hospital in a moribund condition and died the following morning. Postmortem examination, performed 4 hours after death by Dr. A. Lara, Director of the Hospital, and Dr. Diego Hernandez, a representative of the Department of Public Health and Charities of Mexico, established the diagnosis of yellow fever. Blood from the heart and pieces of liver and kidney were obtained for experimental purposes. The details of the experiments will be given later under the heading Results obtained with Case 1.

As soon as the diagnosis of yellow fever had been established in Case 1, Dr. Hernandez ordered the soldiers (eleven in all) who had been quartered with this patient to the yellow fever hospital, there to be placed under quarantine. Among these eleven was a young soldier, A., who was apparently convalescing from a recent attack of yellow fever. The urine and serum of this individual were studied later and will be referred to in the report as Case 3.

On December 18 one of the exposed soldiers, M., began to complain of an illness which suggested strongly the beginning of yellow fever. A systematic study of this case was started at once, the blood being withdrawn from the median basilic vein at first daily and then every other day (December 18, 19, 20, 22, and 24) and used on the one hand for cultivation and on the other, if possible, for inoculation into guinea pigs. As Chart 7 shows, the fever became typical within 3 days, with the development gradually of all other symptoms, congested conjunctivæ, flushed face, muscular pains in the limbs and loins,

[8] Dr. Kligler arrived Dec. 12, 1919, Dr. Noguchi Dec. 23.

severe headache, swollen gums, nausea, epigastralgia, albuminuria, and cylindruria. On December 24 there occurred the characteristic "coffee-grounds" vomit, also definite jaundice, which became much deeper within the next several days. The albumin and casts gradually increased in the urine, and there was an abundance of bile pigment, the amount of urine being also diminished. The patient was discharged at the end of 3 weeks. Before discharge the serum and urine were collected for further study. This case will be referred to as Case 2.

RESULTS OBTAINED WITH CASE 1.

Case 1.—J. M., soldier; brought to the Casa de Salud (the yellow fever hospital) from the barracks in the night of Dec. 15, 1919, in a critical condition, with characteristic black vomit and jaundice. Death occurred at 6 a.m., and autopsy was made at 10 a.m., 4 hours post mortem.

Autopsy.—There was general jaundice, which was particularly marked in the scleras. The face was covered with black vomit. The liver was dotted with small yellowish areas. There were petechial hemorrhages in the stomach wall; the kidneys were hemorrhagic; the pericardium contained yellowish fluid; the serum was icteric.

Blood was drawn from the heart, and a portion of the liver and kidney was removed at autopsy for the experiments to be described. For the purpose of transmission guinea pigs were used throughout the present work.

Transmission Experiments with the Blood, Liver, and Kidney of Case 1.

Blood.

On December 16, 1919, intraperitoneal inoculation of 1 cc. of the heart's blood[9] was made into two guinea pigs (Nos. 1 and 2). Both had a temporary rise of temperature after 7 days but returned to normal within a few days. No jaundice developed at any period. On examination after 18 days there were some suspicious lesions in the lungs, otherwise the findings were negative.

[9] The same specimen of blood kept at 8°C. for 8 days was inoculated into Guinea Pigs 18 and 35 intracardially, but neither symptoms nor high temperature were seen to develop within 2 weeks (negative).

Kidney.

An emulsion of the kidney in sterile saline solution was injected on December 16 into two guinea pigs, one of which showed a very suggestive reaction (Guinea Pig 3).

Guinea Pig 3 (Chart 1).—Dec. 16, 1919. Intraperitoneal inoculation of 1 cc. of the kidney emulsion. Temperature rose to 103° F. in the afternoon of Dec. 19 and was 103° a.m. and 104.6° p.m. on Dec. 20. The animal was killed for transfers to three new guinea pigs on Dec. 21.

Transfers from Guinea Pig 3 (Second Generation).—December 21. 1 cc. of an emulsion prepared from the liver and kidney from Guinea Pig 3 was inoculated intraperitoneally into each of two normal guinea pigs, and 1 cc. of the heart's blood into a third. One died early of a secondary infection, and another remained well after having shown a slight elevation of temperature, while the third animal (Guinea Pig 3c) had definite signs of the leptospira infection.

Guinea Pig 3c (Chart 2).—Inoculation of 1 cc. of the blood of Guinea Pig 3. This animal began to show fever (103.2°) 5 days after the inoculation. The following morning the temperature remained at 103.2° and in the afternoon reached 105.6°. The animal was killed for examination and transfer.

Autopsy.—There was a light yellowish color throughout the subcutaneous tissues. The lungs showed numerous hemorrhagic foci, the gastric mucosa was highly congested, but there were no macroscopic hemorrhages in the latter. The intestines were icteric and congested, the liver was slightly icteric, the kidneys and adrenals were moderately congested, and the spleen was normal. The urine was slightly turbid and contained a considerable amount of albumin (++) and a few casts.

The blood from the heart of Guinea Pig 3c, as well as emulsions of the liver and kidney, were used to inoculate nine new guinea pigs. Two of the three inoculated with the blood and three of the six inoculated with the mixture of emulsions of the liver and kidney succumbed to a secondary infection with the paratyphoid bacillus. Two, however, of those escaping the secondary infection showed temperature curves and lesions quite typical of *Leptospira icteroides* infection, as described below.

Transfers from Guinea Pig 3c (Third Generation).—

Guinea Pig 54 (Chart 3).—Dec. 27, 1919. Received 1 cc. of the heart's blood from Guinea Pig 3c intraperitoneally. The temperature rose on the 7th day, and the animal remained febrile for 48 hours. It was killed for transfer and examination 10 days after the inoculation.

Autopsy.—The lungs showed numerous hemorrhagic foci. The gastrointestinal mucosa was highly congested. The liver was pale, perhaps slightly yellowish, with granular surface and several hemorrhagic spots. The kidneys were apparently swollen and degenerated, the adrenals were small and hemorrhagic, and the spleen was swollen.

Guinea Pig 59 (Chart 4).—Dec. 27, 1919. Received 1 cc. of a mixture of the liver and kidney emulsion from Guinea Pig 3c intraperitoneally. The temperature rose to 104° F. on the 6th day, and was 103° a.m. and 105° p.m. the following day. The animal was killed for transfer and examination on that day.

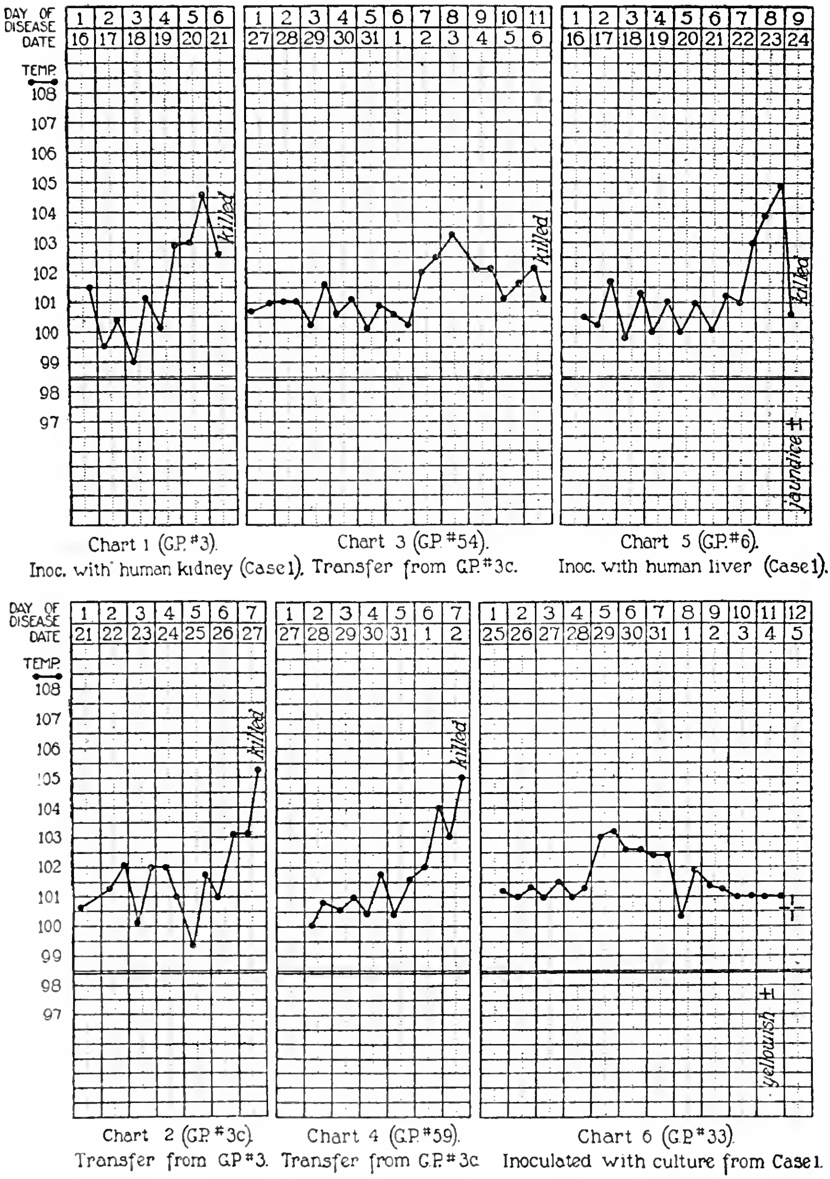
Autopsy.—There was a slight yellowness throughout the body, especially on the abdominal wall; no subcutaneous or intramuscular hemorrhages. The lungs showed several petechial hemorrhages on the posterior surface; there was a general congestion of the lobes. The liver was slightly icteric. The gastrointestinal tract showed scattered hemorrhagic areas, especially in the mucosa of the large intestine. The kidneys were highly congested, the spleen was normal, and the urine icteric.

Guinea Pigs 54 and 59 undoubtedly represented a positive transmission. The heart's blood and emulsions of the kidney and liver of Guinea Pig 59 were inoculated intraperitoneally into six normal guinea pigs, but the animals were lost through secondary bacillary infection.

Liver.

A small piece of the liver from Case 1 was emulsified in a sterile mortar with saline solution, and 1 cc. of the emulsion was inoculated into each of two guinea pigs (Nos. 5 and 6) intraperitoneally on December 16, 1919. One of them (Guinea Pig 6) presented a definite picture of the leptospira infection, while the other gave a suggestive reaction only. The protocol of Guinea Pig 6 and details regarding further transfers are given below.

Guinea Pig 6 (Chart 5).—Dec. 16, 1919. Received 1 cc. of the liver emulsion intraperitoneally. As the chart shows the temperature rose sharply (103° F.) in the afternoon of the 7th day and still more the next day (104° a.m. and 104.9° p.m.). On the 9th day it fell to 100.5° with the simultaneous appearance of a



CHARTS 1 to 6. Transmission experiments with autopsy material from Case 1.

trace of jaundice in the scleras and abdominal wall. The animal was killed for transfer and examination.

Autopsy.—Faint jaundice throughout the body. Lungs showed a few ecchymoses and infarctions. Liver congested, with numerous subcapsular hemorrhagic mottles, perhaps more brownish than normally. Stomach and intestines highly congested. Kidneys and adrenals highly congested, the former showing a parenchymatous degeneration. Spleen normal. Urine turbid, brownish, containing casts and albumin.

Guinea Pig 6 represented a positive transmission, and from this animal transfer was made to three normal guinea pigs (Nos. 19, 27, and 28). The results obtained in these three animals were, first, a definite febrile reaction on the 4th day, followed by an irregular fluctuation of temperature during the several successive days (indicative of a secondary infection) without jaundice at any time. These animals were killed on the 13th day for examination. The lungs showed more or less numerous hemorrhagic foci; the other organs appeared normal. No further passage was made with this material.

Transmission Experiments by Means of Cultures from the Blood of Case 1.

Attempts were made to obtain a culture of *Leptospira icteroides* with the blood obtained from Case 1. Into each of a series of culture tubes containing the medium previously found suitable[10] for the growth of *Leptospira icteroides* 0.1 to 0.5 cc. of the blood was introduced, and the tubes were placed in a thermostat, the temperature being about 26°C. A portion of blood was also mixed with sodium citrate saline solution (equal parts) and after being kept at room temperature (26°C.) for 24 hours was inoculated in amounts of 0.1 to 0.5 cc. into three tubes on December 17, 1919.

Nearly all these tubes were found to be contaminated with a *coli*-like bacillus, owing undoubtedly to a terminal or postmortem invasion by this organism of the blood circulation. On December 25 two culture tubes, inoculated on December 17, appeared to be free from any secondary bacterial contamination. Under the dark-field microscope no leptospira could be found. From each of these two tubes about 1.5 cc. of the uppermost layer of the medium, just beneath

[10] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 13.

the paraffin oil, were taken out by means of small sterile pipettes and the mixture of the two cultures was used for inoculating three guinea pigs. Two of the animals died of a secondary infection within 2 days, and the third (Guinea Pig 33) was found dead on the 12th day after having had slight fever from the 5th to 7th day. The protocol of this animal is given below.

Guinea Pig 33 (*Chart 6*).—Dec. 25, 1919. Inoculated intraperitoneally with 1 cc. of the mixture of Culture Tubes 1 and 2 which had been kept 8 days at room temperature after inoculation with citrated blood from the heart of Case 1 at autopsy. The temperature rose to 103° F. a.m. and 103.2° p.m. on the 5th day, and was 102.6° and 102.4° respectively for the 2 following days. It returned to normal on the 10th day, and death occurred during the night of Jan. 4, 1920, 11 days from the time of inoculation.

Autopsy.—The cadaver had undergone considerable postmortem decomposition, owing to the warm temperature of the laboratory, when found lying dead in the cage on Jan. 5. Notwithstanding postmortem discoloration, there was a slight but distinct yellowness in the skin and scleras. On opening the body also the postmortem changes greatly interfered with the efforts to determine any characteristic lesions. The lungs were congested and showed a few old hemorrhagic areas. There was a small localized abscess on the abdominal wall, and the peritoneal cavity contained some turbid fluid.

It is difficult to determine whether or not the temperature curve and lesions in Guinea Pig 33 were due wholly to the abscess or in part to a mild leptospira infection.

RESULTS OBTAINED WITH CASE 2.

Case 2 (*Chart 7*).—P. M., soldier; exposed to infection through the preceding case. Dec. 17, 1919, 5 p.m. Complained of headache. Dec. 18. Increase in general malaise; headache; rachialgia; pains in the limbs. Blood was drawn at 4 p.m. Temperature 100.9°; pulse 96. No albumin in urine. Dec. 19. Temperature 101.5°; pulse 92. Blood drawn in the morning; citrated and inoculated into two guinea pigs. Trace of albumin. Dec. 20. Temperature 103.5°. Blood drawn and inoculated into three guinea pigs. Albumin 0.05 gm. Dec. 21. Trace of albumin. Dec. 22, 3 p.m. Temperature 99.3°; pulse 78. Conjunctiva injected; scleras slightly icteric. Vomited. Stool chocolate color. Urine turbid; albumin 0.18 gm. Blood drawn for culture. Dec. 23. Albumin 1.4 gm. Pulse 72. Dec. 24. Albumin 1.8 gm.; casts and renal epithelium. Jaundice marked. Blood drawn for culture and animal inoculations. Pulse 72. Dec. 25. Albumin 2.2 gm. Dec. 26. Albumin 5.5 gm. Dec. 27. Albumin 0.8 gm.

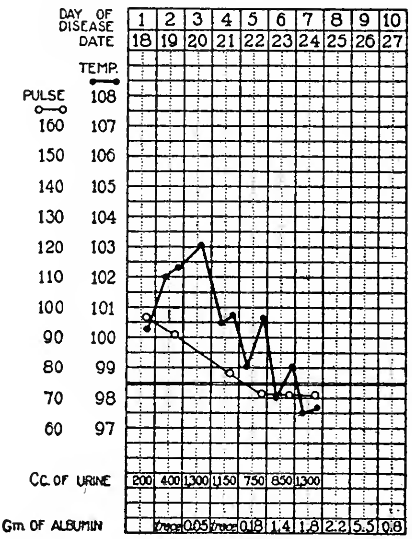


Chart 7 (patient).
Temperature curve, Case 2.

Transmission Experiments with Blood of Case 2.

First Specimen of Blood, Drawn on the 2nd Day of the Disease, December 19, 1919.

1 and 2 cc. respectively of the citrated blood were inoculated intraperitoneally into two guinea pigs (Nos. 7 and 8).

Guinea Pig 7.—The animal had a temperature of 103° F. on the 5th day; during the following 48 hours it was normal but rose again to 102.8–102.5° for 3 succeeding days. On the 12th day it was normal. On the skin of the abdominal wall were several ecchymotic areas. The animal was killed for examination.

Autopsy.—With the exception of one small hemorrhagic spot in the lungs nothing abnormal was found.

Notwithstanding this apparently negative finding, however, the blood, as well as emulsions of the liver and kidney of this animal, were inoculated into six normal guinea pigs, the blood into one and the mixed emulsions of the liver and kidney into five. The results were of doubtful nature with respect to positive transmission, although two of these animals showed some punctiform hemorrhages in the lungs when examined after 14 days.

Guinea Pig 8 (*Chart 8*).—This experiment was a duplicate of the experiment with Guinea Pig 7; that is, the animal was inoculated with the blood taken on

Dec. 19, 1919. As Chart 8 shows, the temperature rose to 104° F. in the afternoon of the 4th day, remaining high for the following 24 hours. It fell to 100.5° on the 6th day, when it seemed advisable to make a transfer.

Autopsy.—The examination showed a few hemorrhagic foci in the lungs, highly congested liver and kidneys, and a few hemorrhagic areas in the mucosa of the intestines.

Transfers with the blood and organ emulsions (liver and kidneys) of Guinea Pig 8 were made to several normal guinea pigs.

Transfers from Guinea Pig 8 (Second Generation).—

Guinea Pig 20 (Chart 9).—Dec. 24, 1919. Inoculated with the citrated blood from Guinea Pig 8, 1 cc. intraperitoneally. The temperature rose suddenly to 105° on the 5th day and remained at that point during the day. It fell to 103° the next day and to 101° on the 8th day. At this period the scleras seemed slightly icteric. The urine diminished to less than 2 cc. and contained albumin. The condition remained unchanged for the 3 succeeding days, the urine still containing albumin. Casts appeared on the 10th day. The yellowness in the conjunctiva was definite on the 11th day, and the animal died in the afternoon after subnormal temperature.

Autopsy.—Small hemorrhagic and congested areas in the lungs; liver smaller in size (?) and reddish brown with yellow mottles; kidneys unusually pale, and the demarcation between cortex and medulla indistinct. The spleen was somewhat enlarged, perhaps owing to secondary infection, and granular. In the liver and kidney stained by Levaditi's method fairly numerous leptospiras were demonstrated.

Guinea Pig 21.—Dec. 24, 1919. Inoculated intraperitoneally with 0.5 cc. of the mixture of the liver and kidney emulsions of Guinea Pig 8. Died of a secondary infection, but showed the presence of a mild leptospira infection, with albumin and casts in the urine.

Guinea Pig 22.—This experiment was a duplicate of the foregoing experiment. Temperature 103° F. on the 4th day, and fever continued for several days. Jan. 5, 1920. The animal was killed for examination.

Autopsy.—The lungs showed old hemorrhagic areas, but the other organs seemed normal.

The remainder of the citrated blood of December 19 was preserved in the ice box (about 10°C.) and after 5 days was inoculated intracardially into two normal guinea pigs, with negative results.

Second Specimen of Blood, Drawn on the 3rd Day of the Disease, December 20, 1919.

1 cc. of the citrated blood drawn on December 20 was inoculated intraperitoneally into three guinea pigs (Nos. 9, 10, and 11) on the same day.

Guinea Pigs 9 and 11.—These two animals had suggestive and almost identical temperature curves.

Autopsy.—When killed for examination on the 15th day, there were several foci of recent hemorrhage in the lungs, and the kidneys were congested; the other organs appeared normal.

Guinea Pig 10 (Chart 10).—This animal presented a more definite febrile reaction. On the 14th day it had a normal temperature and appeared well. It was killed for examination on that day.

Autopsy.—There were numerous hemorrhagic foci in the lungs; the abdominal wall and subcutaneous tissues were icteric and showed several large hemorrhagic areas. The adrenals were hemorrhagic, the kidneys highly congested, and the liver was apparently normal. There were pin-point hemorrhages in the gastric mucosa, and hemorrhages in the intestinal mucosa. The spleen appeared normal.

The remainder of the citrated blood of December 20 was preserved in the ice box (about 10°C.) for 4 days and 1 cc. was inoculated intracardially into each of two normal guinea pigs (Nos. 14 and 15).

Guinea Pig 14.—Sudden rise of temperature to 104° F. in the afternoon of the 4th day, but it dropped to normal (102°) on the next day. On the 6th day there was another rise to 103.5°, from which the temperature gradually fell to subnormal on the 8th day. The urine gradually diminished in quantity after the 4th day, and albumin and casts were present for several days. Ecchymotic hemorrhages were observed on the abdominal wall on the 8th day. There was a suspicion of jaundice. The animal rapidly recovered and was normal on the 13th day.

Guinea Pig 15.—Lost through secondary infection.

Third Specimen of Blood, Drawn on the 5th Day of the Disease, December 22, 1919.

The citrated blood from Case 2 drawn on December 22 was kept on ice for 48 hours (about 10°C.) before it was inoculated into two guinea pigs (Nos. 16 and 17) on December 24.

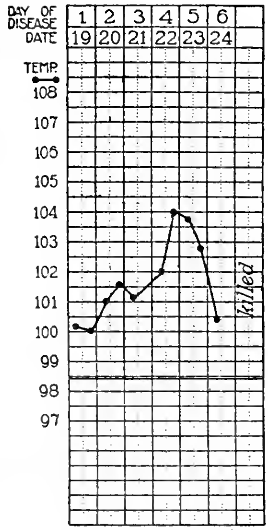


Chart 8 (G.P. #8).
Inoc. with 2nd day blood.

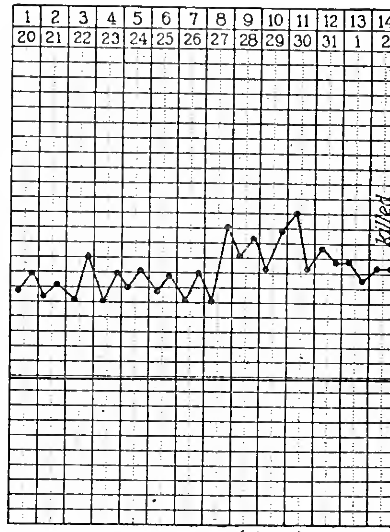


Chart 10 (G.P. #10).
Inoculated with 3rd day blood.

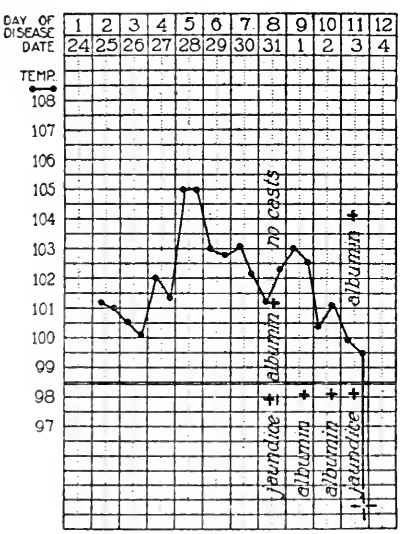


Chart 9 (G.P. #20)
Transfer from G.P. #8

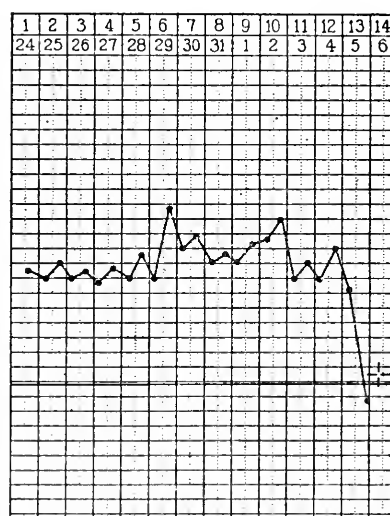


Chart 11 (G.P. #25)
Inoculated with 7th day blood.

CHARTS 8 to 11. Direct transmission experiments with blood from Case 2.

Guinea Pig 16.—Showed a rise of temperature to 103° F. on the 4th day, a decline to 102.5° on the 5th day, and a rise again to 103.5° on the 6th. At this time the animal had congested conjunctivæ. On the 9th day the temperature became somewhat subnormal but soon returned to normal during the succeeding days. The animal was killed on the 13th day.

Autopsy.—A number of hemorrhagic foci were found in the lungs, the liver appeared very pale and brownish, the kidneys were somewhat congested, the spleen normal. The animal was probably recovering from an abortive infection.

Guinea Pig 17.—This animal had practically the same febrile reaction as Guinea Pig 16.

Autopsy.—Performed on the 13th day. The organs appeared normal, with the exception of a few hemorrhagic foci in the lungs.

Fourth Specimen of Blood, Drawn on the 7th Day of the Disease, December 24, 1919.

The citrated blood was intracardially inoculated into three guinea pigs (Nos. 24, 25, and 26) within 3 hours from the time of withdrawal, each animal receiving 0.2 to 0.5 cc.

Guinea Pigs 24 and 25 (Chart 11).—Both animals had a suggestive temperature curve but had no jaundice at any period.

Autopsy.—The lungs showed some suspicious recent hemorrhagic areas; the kidneys were rather congested. The other organs were normal. Results inconclusive.

Guinea Pig 26.—Perhaps died of secondary infection.

Transmission Experiments by Means of Cultures from the Blood of Case 2.

Cultures were made with the citrated blood of Case 2 by introducing into a series of six tubes containing the necessary culture media[11] quantities of the blood varying from 0.1 to 0.5 cc. and adding to each a layer of paraffin oil. The tubes were kept for several days at a temperature of 26–28°C. Then, since the leptospira grows only in the presence of oxygen, the upper layer of the semisolid medium was removed from each tube by means of a sterile pipette and the contents of two or more tubes were mixed in a Petri dish. Several guinea

[11] This consisted of a rabbit serum diluted three times with 0.9 per cent sodium chloride solution and rendered semisolid by the addition of 0.1 to 0.3 per cent nutrient agar (melted). Each tube contained about 6 cc. of the mixture.

pigs were inoculated with the same material. For every series of cultures made from any given specimen of blood at least two sets of guinea pigs were inoculated, and three sets if there was a larger number of tubes.

The cultures set up with the specimens of blood taken on five occasions (December 18, 19, 20, 22, and 24) remained for the most part free from any secondary contamination during the first 5 days. Shortly afterwards, however, darkening of the hemoglobin in several tubes indicated the presence of some sort of contamination, and microscopically a fungus growth was found. Unless a surface growth of fungus was already evident, however, the contents of these tubes were also used for inoculation, though tubes showing bacterial contamination were discarded. Such contamination was rarely met with in experiments with the blood of Case 2, and that occasionally occurring was probably due to technical error. Following are the results of animal inoculations with the various culture series.

Culture, First Series. Blood Drawn on the 1st Day of the Disease, December 18, 1919.

Dark-field examination of Tubes 1 and 2, 7 days after the cultures had been set up, revealed no organism. The contents of these two tubes (2 cc. of the uppermost layer of medium from each) were mixed together in a Petri dish, and 1 cc. of the mixture was inoculated intraperitoneally into each of three normal guinea pigs (Nos. 36, 37, and 38). No definite reaction followed the inoculations in Guinea Pigs 36 and 38, which, on examination 15 days after inoculation, presented nothing remarkable in any organ except for a congestion of the lungs.

Guinea Pig 37.—Slight rise in temperature on the 7th day (102.5° F. a.m., 103° p.m.), with return to normal the next day. On the 14th day the temperature rose suddenly to 105° a.m. and 103° p.m.; on the 15th day it was 102.6° a.m. The animal was killed the same day for examination.

Autopsy.—The lungs were typical, showing numerous hemorrhagic foci, but there was no other change.

Dark-field examination of Tubes 3 and 4 of the same series demonstrated the presence of a few fungi on December 25, 7 days after

inoculation. The uppermost layers of media from the two tubes were mixed and 1 cc. of the mixture was inoculated intraperitoneally into two guinea pigs (Nos. 39 and 40) on that day (December 25), with negative results.

Culture, Second Series. Blood Drawn on the 2nd Day of the Disease, December 19, 1919.

Five culture tubes were available for these experiments. These were divided into two groups, Tubes 1 and 2, and Tubes 3 to 5. Dark-field examination of these tubes was made 6 days after the inoculation. Tubes 1, 2, 3, and 5 showed no organism; Tube 4 showed a few immotile leptospiras, some apparently degenerating. Occasional coarse, short filaments (fungus) were also found. The number of leptospiras was so small that a repeated, thorough search with a powerful illumination was necessary to find one. The results of animal inoculation follow.

The contents (uppermost layers) of Tubes 1 and 2 of the second culture series were taken out and mixed. 1 cc. of the mixture was inoculated intraperitoneally into two guinea pigs (Nos. 41 and 42) on December 25 and into two more on December 26. Two died of secondary infection; the others remained well.

The uppermost layer was taken out of Tubes 3, 4, and 5 of the second culture series, and 1 cc. of the mixture of these, which had been found by dark-field examination to contain a few leptospiras, was inoculated intraperitoneally into each of three guinea pigs (Nos. 43, 44, and 45) 6 days after the cultures had been made. The results of these inoculations were all positive, as the protocols show.

Guinea Pig 43 (Chart 12).—The temperature rose to 103° in the morning of the 5th day. During the next 36 hours it fluctuated between 102° and 102.5°. On the 7th day it rose to 105.2° a.m. and 103.5° p.m. The amount of urine was still undiminished. On the 8th and 9th days the morning temperature was 102° and the evening 104°; on the 10th it was 102.5° in the morning and 103.4° in the evening; on the 11th it fell rapidly to normal. The animal was decidedly jaundiced. The urine diminished daily after the 9th day, and only 3 cc. were secreted during the 24 hours from Jan. 3 to Jan. 4, 1920. Albumin and casts were present. The animal was killed for examination and transfer on the 11th day.

Autopsy.—Findings typical. Intense jaundice; extensive subcutaneous ecchymoses; lungs distinctly hemorrhagic; gastrointestinal mucosa congested with

hemorrhagic areas; liver yellowish; kidneys hemorrhagic and yellowish; spleen somewhat enlarged.[12] Dark-field examination revealed the presence of leptospiras in the blood as well as in emulsions of the liver and kidney.

Transfers from Guinea Pig 43.—Blood from the heart and a mixture of emulsions of the liver and kidney were intraperitoneally inoculated into six normal guinea pigs, the blood into Nos. 106 and 107, and the emulsion into Nos. 108, 109, 110, and 111. The protocols follow.

Guinea Pig 106.—Jan. 4, 1920. Inoculated with 1 cc. of the citrated blood from Guinea Pig 43. Temperature 104° F. on the 3rd day, fell to normal on the 4th, rose to 104° a.m., 104.5° p.m. on the 5th, remaining high for another 24 hours. On the 7th day it was 103.5° a.m. and 102.2° p.m. Jaundice and albuminuria were both distinct. On the 8th day died after subnormal temperature.

Autopsy.—All the characteristic lesions, together with signs of a secondary infection with the paratyphoid bacillus (enlarged spleen and fibrinous exudate in the peritoneal cavity).

Guinea Pig 107 (*Chart 13*).—This experiment was a duplicate of the foregoing experiment. As shown in Chart 13 the course of the infection was typical, the temperature rising to 105.2° a.m. and 104.6° p.m. on the 5th day. On the 7th day the animal was intensely yellow, with albumin and casts in the urine. It was killed for examination on the 7th day.

Autopsy.—Findings typical. The blood and organ emulsions showed a few leptospiras.

Guinea Pig 108.—Jan. 4, 1920. Inoculated intraperitoneally with 0.5 cc. of the mixture of the liver and kidney emulsions from Guinea Pig 43. This animal suffered from a secondary paratyphoid infection and showed a temperature of 104.6° in the afternoon of the 3rd day. This early fever endured for 3 days longer. On the 7th day it fell to 103.5°. On the 8th day it reached normal, and the animal died after subnormal temperature on the 9th day. No jaundice developed.

Autopsy.—Several hemorrhagic foci in the lungs. No leptospira was found.

Guinea Pig 109.—This animal must have been suffering from an intercurrent infection before the inoculation, as the temperature was 103.4° in the afternoon of Jan. 3, 1920, a day before the inoculation. After inoculation the temperature fluctuated between 102° a.m. and 102.5° p.m. during the 2 days that followed. On the 4th day it was normal. On the 5th it rose to 103.5° a.m. and 103.2° p.m., on the 6th it was 102.5°, on the 7th 101.5° a.m. and 96° p.m. Jaundice developed on the 6th day.

Autopsy.—Performed on the 7th day. The lesions were typical, hemorrhages in the lungs, stomach, and intestines, kidneys highly hyperemic with minute hemorrhages, liver yellowish. The spleen was enlarged, showing the effect of a

[12] Blood culture showed the presence of the paratyphoid bacillus.

secondary infection with the paratyphoid bacillus. The blood and organ emulsions contained leptospiras. A blood culture from the heart showed the presence of the paratyphoid bacillus.

Guinea Pig 110 (*Chart 14*).—Duplicate experiment. This animal escaped secondary infection with the paratyphoid bacillus and ran a typical course of the leptospira infection. The temperature rose to 103° in the afternoon of the 3rd day, was 102.2° a.m. and 104° p.m. on the 4th, 103.8° a.m. and 104.6° p.m. on the 5th. From the 6th day the temperature rapidly fell and was 98° when death occurred in the morning of the 7th day. Jaundice was definite on the 6th day.

Autopsy.—Findings typical. The leptospira was demonstrated in the organ emulsions. The animal had epistaxis at the time of death.

Guinea Pig 111 (*Chart 15*).—Duplicate experiment. This animal also escaped secondary paratyphoid infection and ran a course of fever similar to that of Guinea Pig 110, except that the highest temperature never exceeded 104° (4th day). On the 5th the temperature was still 103.8°. The examination of the blood showed no leptospiras. The animal was killed for examination and culture in the afternoon of the 5th day. The citrated blood from the heart was examined carefully for the leptospira, and one was found after long search. This blood was used for cultivation, which in 7 days was successfully accomplished.

Autopsy.—Examination of the organs showed that jaundice had not yet developed. The lungs, stomach, and intestines were dotted with hemorrhagic foci. The liver was congested, with yellowish areas. The kidneys were congested. Emulsions of the liver and kidney contained a small number of leptospiras.

Guinea Pig 44 (*Chart 16*).—This is one of the three guinea pigs inoculated with 1 cc. of the mixture of Culture Tubes 3, 4, and 5 of the second culture series on Dec. 25, 1919. This animal, in striking contrast with the foregoing ones (Guinea Pig 43 and its transfers) showed a rather slowly developing infection without much febrile reaction. The temperature remained entirely within the normal fluctuation until the 7th day, and on the 8th day the morning temperature was slightly above normal (102.6° F.), rising to 103.6° in the afternoon. On the 9th day the temperature was normal in the morning and somewhat lower in the afternoon, and the animal was distinctly yellowish. On the 10th day the temperature registered 101.3° a.m. and 101.2° p.m., perhaps somewhat below the normal, and jaundice was intense. The animal died during the night.

Autopsy.—Performed in the morning, Jan. 4, 1920. Intense jaundice. There were extensive subcutaneous hemorrhagic foci on the abdominal wall and in adjacent regions. The lungs were yellowish, with numerous hemorrhagic areas, and the heart was dilated and contained dark clot and blood. The liver was yellowish brown, mottled with congested areas, the gall bladder empty, the kidneys were swollen, hemorrhagic, and degenerated, deeply bile-stained, and the adrenals hyperemic. The stomach contents were bloody, and there were ecchymoses in the mucosa. The intestines contained bloody stools; the mucosa was congested and showed many hemorrhagic spots. The spleen was normal. The

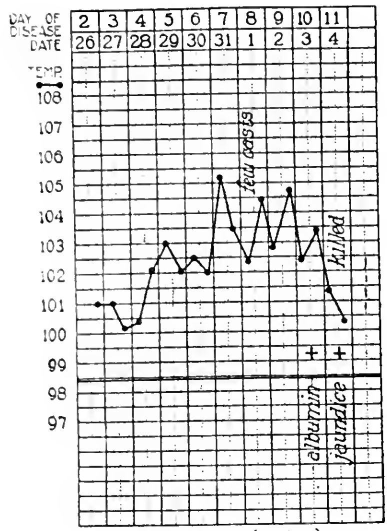


Chart 12 (G.P.#43).
Inoc. with culture of 2nd day blood.

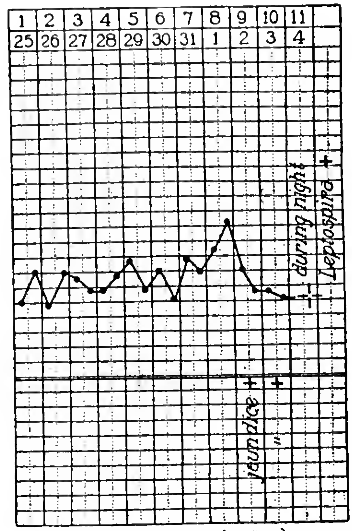


Chart 16 (G.P.#44).
Inoc. with culture of 2nd day blood.

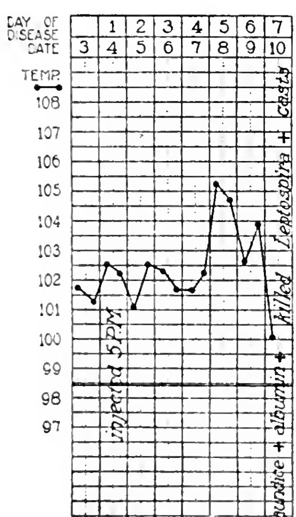


Chart 13 (G.P.#107).
Transfer from G.P.#43.

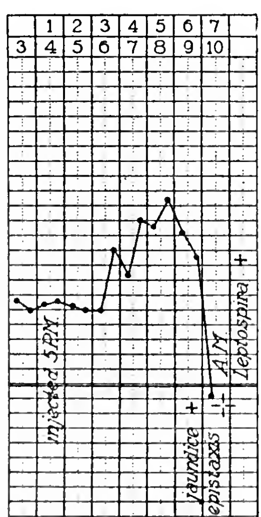


Chart 14 (G.P.#110).
Transfer from G.P.#43.

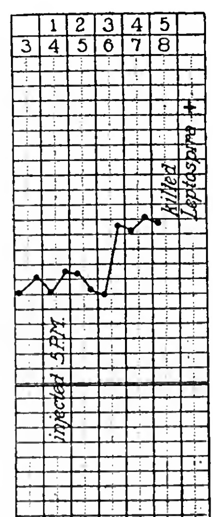


Chart 15 (G.P.#111).
Transfer from G.P.#43.

CHARTS 12 to 16. Transmission experiments with cultures from Case 2.

bladder was almost empty (anuria); ovaries and uterus congested. In the scanty urine removed from the bladder there were albumin and numerous casts with erythrocytes. The leptospira was found in the blood.

Transfer with a mixture of the liver and kidney emulsions from Guinea Pig 44 was made into Guinea Pig 105 on January 4, 1920. The result was positive in every respect. The animal succumbed to a typical leptospira infection on the 7th day, and the leptospira was detected in the blood and organs.

Guinea Pig 45.—This is the last of the three guinea pigs (Nos. 43, 44, and 45) inoculated with the same material (culture made with the blood drawn on the 2nd day) on Dec. 25, 1919. The result of the inoculation was likewise positive, although the temperature curve deviated considerably from the curves of the other two animals, owing probably to a secondary infection with another organism. On the 11th day the animal had been running a temperature of 103–104° F. for 3 days. It was killed for examination on the 11th day.

Autopsy.—The lesions were typical except for the absence of distinct jaundice. The liver was, however, somewhat yellowish. The leptospira was not found by routine dark-field examination.

Culture, Third Series. Blood Drawn on the 3rd Day of the Disease, December 20, 1919.

The citrated blood of Case 2 drawn on December 20 was introduced into five culture tubes and incubated for 5 days at 26°C. Tubes 1, 2, and 5 were free from any contamination, while Nos. 3 and 4 showed a fungus growth. A search for the leptospira by dark-field examination showed occasional immotile leptospiras in the contents from Tube 4, but was unsuccessful in the case of the other tubes.

The uppermost layer of culture medium from Tubes 1 and 2 was taken out and the two were mixed, as was done also with Tubes 3, 4, and 5. The two mixtures were inoculated into corresponding groups of guinea pigs.

Guinea Pigs 46 and 47.—Inoculated with 1 cc. of the mixture of Culture Tubes 1 and 2. Both animals had a suggestive temperature curve. On the 13th day both guinea pigs were killed for examination.

Autopsy.—Findings similar, both animals having old hemorrhagic foci in the lungs, congested stomach and kidneys, and normal spleen. The liver was slightly yellowish in Guinea Pig 46.

There was perhaps an abortive infection in both these animals.

Three guinea pigs (Nos. 48, 49, and 50) were inoculated each with 1 cc. of the mixture of Culture Tubes 3, 4, and 5. The results were highly suggestive of an abortive leptospira infection with Guinea Pigs 48 and 49, and quite definite with Guinea Pig 50, in which jaundice was also present. From Guinea Pig 48 a positive transfer to another guinea pig was also accomplished.

Guinea Pig 48.—Typical temperature rise on the 5th day, reaching 103.8° F. a.m. and 103.4° p.m. The temperature remained normal on the 6th day and continued so until the 9th day, when the animal was killed for examination and transfer.

Autopsy.—The lesions found were small, discrete hemorrhagic foci in the lungs, swelling and congestion of the kidneys, and congestion of the stomach mucosa. The other organs appeared to be normal.

Transfers were made with the citrated blood from the heart of Guinea Pig 48 into two guinea pigs and with a mixture of the liver and kidney emulsions into four guinea pigs. One of the animals inoculated with the blood showed a temperature of 102.8°F. in the afternoon of the 8th day and 103° in the morning of the 9th day, but soon returned to normal. When it was killed for examination on the 12th day there were a few hemorrhagic patches in the lungs, the liver was somewhat yellowish, and the kidneys were congested.

Transfer with the organ emulsions from Guinea Pig 48 gave a more decided picture of the leptospira infection in some but no reaction in others.

Guinea Pig 98.—Temperature of 102.8° on the 5th day, 102.5° a.m. and 103.2° p.m. on the 6th, 103° a.m. and 102° p.m. on the 7th. On the 9th the temperature became normal. The animal was killed on the 12th day for examination.

Autopsy.—The lungs showed many recent hemorrhagic patches, the mucosa of the stomach several old hemorrhagic foci. The kidneys were pale; the spleen was normal.

Culture, Fourth Series. Blood Drawn on the 5th Day of the Disease, December 22, 1919.

Only four tubes were made with the citrated blood drawn on December 22 from Case 2, and after 9 days only two tubes appeared to be free from bacterial contamination. These tubes also contained a slight fungus growth which did not change the appearance of the

medium to any marked extent. Under the dark-field microscope two undoubted immotile leptospiras were found in the mixture of the contents of the two tubes. 1 cc. of the mixture was also inoculated into each of four guinea pigs.

The results of this series of inoculations were unsatisfactory, owing to a secondary infection in all the guinea pigs.

Culture, Fifth Series. Blood Drawn on the 7th Day of the Disease, December 24, 1919.

Six culture tubes were inoculated with the citrated blood of this date. Unfortunately the tubes were covered with an accidentally contaminated paraffin oil, and within a few days incubation at 28°C. all of them contained a profuse growth of fungus.

SUMMARY.

Injections into guinea pigs of the blood and the emulsions of liver and kidney obtained at autopsy from a fatal case of yellow fever in Merida induced in some of these animals, after a period of several days incubation, a rise of temperature which lasted 1, 2, or more days. When killed for examination at this febrile stage the animals invariably showed hemorrhagic areas of various size, sometimes few and sometimes numerous, in the lungs, and also, though less constantly, in the gastrointestinal mucosa, together with general hyperemia of the liver and kidneys. In a guinea pig (No. 6) inoculated with the liver emulsion of Case 1 there was a trace of jaundice on the 9th day. Injections of the blood or liver and kidney emulsions from such animals into normal guinea pigs reproduced the febrile reactions and the visceral lesions. The majority of the animals which were allowed to live and complete the course of the infection rapidly returned to normal (within several days). Examinations of these surviving guinea pigs after 2 weeks revealed the presence of rather old hemorrhagic foci in the lungs.

In the course of further attempts to transfer the passage strain, a secondary infection by a bacillus of the paratyphoid group caused many deaths among the guinea pigs and resulted finally in the loss of the strain from Case 1.

Most of the cultures made with the heart's blood taken at autopsy from Case 1 proved to be contaminated with a bacillus of the *coli* group. The contents of the apparently uncontaminated tubes were inoculated into guinea pigs, but the results were for the most part negative or vitiated by a secondary infection.

Dark-field search for the leptospira with the autopsy materials was negative, although prolonged and thorough examination was not practicable at the time of these experiments. Our efforts were concentrated on obtaining positive animal transmission rather than on the time-consuming demonstration of the leptospira, which when unsuccessful does not necessarily exclude the presence of the organism in small numbers. Likewise, the dark-field work with the material from guinea pigs was confined to a brief examination and was omitted in many instances. Under these circumstances no leptospira was encountered in any of the material from Case 1.

On the other hand, the results obtained with the specimens of blood from Case 2 were definitely positive, not only in the transmission of the disease directly, or indirectly by means of cultures, into guinea pigs, but also in the demonstration of the leptospira in the primary cultures and in the blood and organ emulsions of guinea pigs experimentally infected with such cultures.

Definite positive direct transmissions were obtained with the specimens of blood drawn on the 2nd and 3rd days. No blood was taken on the 4th or 6th days. There were indications of abortive or mild leptospira infection in the guinea pigs inoculated with the blood taken on the 5th day.

Regarding the inoculation of cultures from Case 2, it may be stated that only the cultures (leptospira +) made with the blood drawn on the 2nd day caused a definite fatal infection in guinea pigs. From this series a continuous passage in the guinea pig has been successfully accomplished. One of the guinea pigs (No. 48) inoculated with the culture 5 days old (leptospira +) made from the blood taken on the 3rd day presented typical symptoms, and a positive transfer from this to another animal (No. 98) was also made. Cultures of the blood drawn on the 5th and 7th days gave unsatisfactory results, owing to a secondary contamination. Leptospiras were detected in some of the culture tubes containing 2nd and 3rd day

specimens of blood from Case 2; they were few in number and for the most part immotile, owing perhaps to some unfavorable cultural condition such as a fungus contamination.

Charts 17, 18, and 19 give a summary of the experiments.

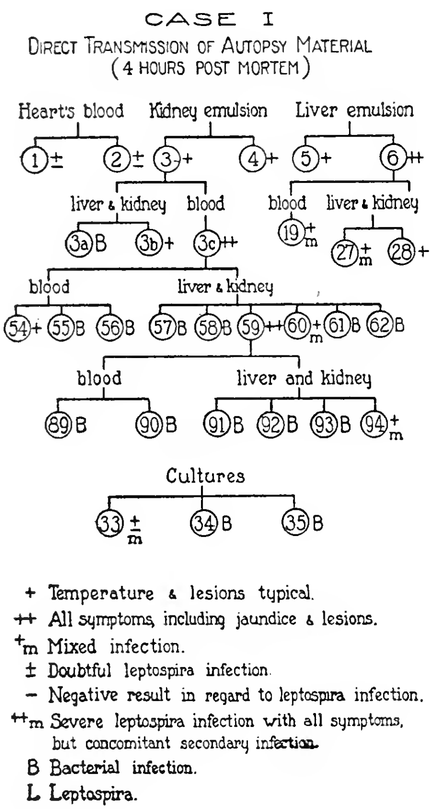


Chart 17.

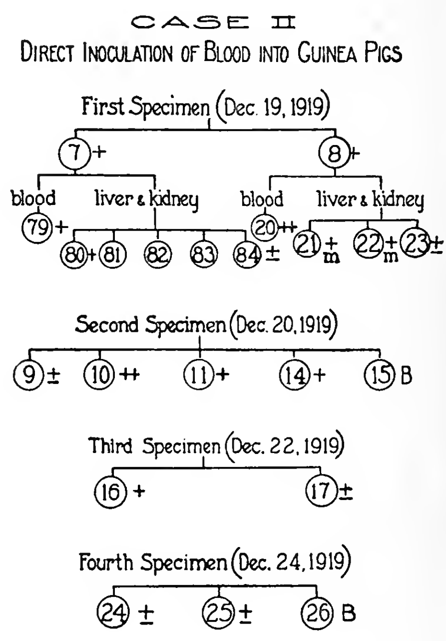


Chart 18.

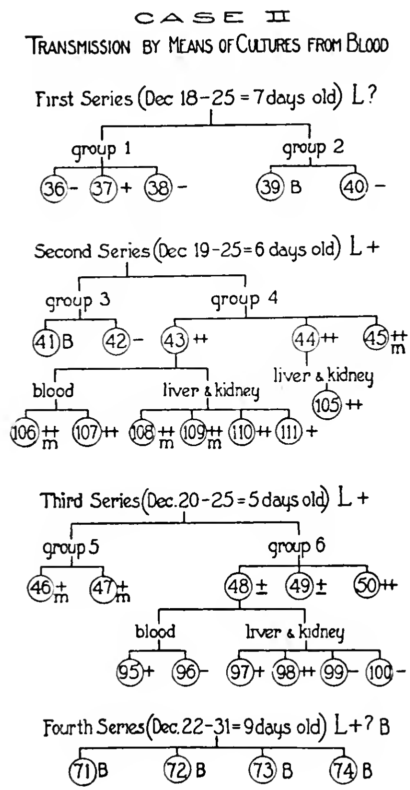

CASE II
Transmission by Means of Cultures from Blood
First Series (Dec 18-25 = 7 days old) L?
group 1
group 2
36 - 37 + 38 -
39 B 40 -
Second Series (Dec 19-25 = 6 days old) L +
group 3
group 4
41 B 42 - 43 ++
44 ++ 45 ++ m
blood
liver & kidney
liver & kidney
105 ++
106 ++ m 107 ++ 108 ++ m 109 ++ m 110 ++ 111 +
Third Series (Dec. 20-25 = 5 days old) L +
group 5
group 6
46 ± m 47 ± m
48 ± 49 ± 50 ++
blood
liver & kidney
95 + 96 - 97 + 98 ++ 99 - 100 -
Fourth Series (Dec. 22-31 = 9 days old) L+? B
71 B 72 B 73 B 74 B

Chart 19.

IMMUNOLOGICAL STUDIES WITH A STRAIN OF LEPTOSPIRA ISOLATED FROM A CASE OF YELLOW FEVER IN MERIDA, YUCATAN.

By HIDEYO NOGUCHI, M.D., AND I. J. KLIGLER, Ph.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, April 3, 1920.)

Morphology, Cultural Properties, and Virulence of the Strain of Leptospira Isolated in Merida.

The morphological features and cultural properties of the strain of *Leptospira icteroides* isolated in Merida are similar to those of the Guayaquil strains.[1] With respect to pathogenicity and virulence we obtained the following data.

Guinea Pig 112 was inoculated on January 4, 1920, with the mixed emulsions of liver and kidney from Guinea Pig 43, which had typical symptoms as a result of inoculation of a culture made with blood drawn from Case 2 on December 19, 1919. No. 112 came down with typical symptoms on the 8th day and was killed for transfer on January 12. The liver and kidney were emulsified together with 0.9 per cent saline solution, about 1 gm. of organ material to 10 cc. of saline solution. The emulsion was allowed to stand several minutes until the supernatant fluid was free from coarse tissue particles. The clear portion, which under the dark-field microscope showed a few leptospiras in every field, was used to determine the minimum lethal dose for guinea pigs. The procedure consisted in inoculating intraperitoneally graduated amounts of the emulsion into as many guinea pigs as the number of dilutions required, each amount being contained in a uniform volume of 1 cc. of 0.9 per cent saline solution. The results of the experiment are recorded in Table I.

The period of incubation varied from 3 to $7\frac{1}{2}$ days, and death occurred 7 to 10 days after the time of inoculation. The duration

[1] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 13.

of illness, calculated from the beginning of fever until death, varied from 2½ to 4½ days. We did not succeed in finding the minimum lethal dose, which apparently lies below 0.0001 cc., the smallest quantity of emulsion employed in the present experiment.

The Merida strain showed a marked pathogenicity for young pups, 6 weeks old, when given intraperitoneally.[2]

TABLE I.

Determination of the Minimum Lethal Dose of the Merida Strain.

Guinea pig No.	Amount of organ emulsion.	Length of incubation.	Time of death.
	cc.	*days*	*days*
163	0.5	3	7
164	0.2	3½	8
165	0.1	3½	7
166	0.01	5	8
167	0.001	7½	10
168	0.0001	5	9

Dog 1.—Jan. 14, 1920. Inoculated with 4 cc. of the kidney emulsion from Guinea Pig 102 (second passage in guinea pigs) intraperitoneally. The temperature was 39.6°C. on the 4th day, falling rapidly to 38° the next morning. In the afternoon of the 7th day the animal was found near collapse (37°) and was killed for examination. Jaundice had been noticed in the conjunctivæ on the 6th day, and there was general jaundice on the 7th day. Albuminuria began on the 5th day and increased until the animal was killed.

Autopsy.—General icterus; marked congestion of the lungs and other organs. Hemorrhagic foci were found in small number in the lungs and intestinal mucosa. The kidneys were congested and degenerated. The liver was mottled with yellowish patches. No subcutaneous or intramuscular hemorrhages were found. The serous membranes were free from hemorrhage.

Dog 2.—This animal received the same material as Dog 1 at the same time. The temperature curve was somewhat more irregular, 39°C. on the 2nd and 3rd days, normal on the 4th day, and 39.6° on the 5th day. The decline was very rapid, 37.5° a.m. and 36.2° p.m. on the 6th day. The animal died during the night. Albuminuria was present on the 4th day. Jaundice was noticeable on the 5th day and had definitely increased during the 6th day.

Autopsy.—General jaundice; liver and other organs yellowish. Few small hemorrhagic foci in the lungs. The stomach contained some digested blood,

[2] Villamil Mendoza, M., Apuntes acerca de la fiebre amarilla, Thesis, Merida, 1920.

and the mucosa of the intestines was profusely hemorrhagic. The kidneys were congested and degenerated. Subcutaneous tissues, muscles, and serosa not hemorrhagic.

Identification of the Merida Strain and the Therapeutic Value of Anti-icteroides Serum against This Strain.

The identity of the Merida strain of the leptospira was established by means of a series of experiments in which varying quantities of anti-*icteroides* serum were injected, together with 1 cc. of an emulsion of the liver and kidney from Guinea Pig 43, into several normal

TABLE II.

Identification of the Merida Strain and the Therapeutic Value of Anti-icteroides Serum against This Strain.

Guinea pig No.	Amount of serum.	Result.	Lesions.
	cc.		
109	0 (control).	Killed after 6 days.	Typical.
110	0 (").	Died " 6 "	"
111	0 (").	Killed " 4 "	" lung lesions.
112	0.1	Survived.	
113	0.01	"	
114	0.001	"	
115	0.0001	"	

guinea pigs. The anti-*icteroides* serum had been prepared in a horse by repeated injections of different strains of *Leptospira icteroides* from Guayaquil cases. The results are recorded in Table II.

The outcome of this experiment made it evident that the Merida strain belongs to the same group as the Guayaquil strain.

Polyvalent anti-*icteroides* immune serum prepared in the horse or monovalent anti-*icteroides* immune serum prepared in the rabbit had a definite devitalizing action upon the Merida strain, while antiserums similarly prepared with strains of *icterohæmorrhagiæ* had no perceptible effect upon the Merida strain.

Serotherapeutic Experiments.

The purpose of the next series of experiments was to ascertain whether or not the same immune serum possessed a therapeutic value in infection with the Merida strain such as had been demonstrated in the case of experimental infection in guinea pigs with the Guayaquil strains.[3] Thirty-one guinea pigs were inoculated at the same time with 0.5 cc. of the organ emulsion, representing at least 5,000 minimum lethal doses. 1 hour after inoculation two of the animals were given an injection of 0.0001 cc. of the serum, and two more received an injection of 0.1 cc. The procedure was continued with each group of four animals after periods of 24, 48, and 72 hours and 4 and 5 days respectively. After 5 days the amounts of serum injected were increased to 1 and 2 cc., as it had been found that the smaller doses had no effect at this period of the disease.

All the animals which received no serum until after 72 hours showed fever at that time; in those untreated until 4 days there were fever and slight jaundice; those not treated until 5 days showed a decline in temperature and increasing jaundice; and in the group which received the injection after 6 days there were intense jaundice and subnormal temperature, and the animals were near or in collapse. The results of this series are summarized in Table III.

0.1 cc., therefore, of the anti-*icteroides* serum prevented any external manifestation of the infection if given before the onset of fever (within 72 hours after inoculation). The same dose, given to animals in the febrile stage, but without jaundice, prevented the development of jaundice; animals still having fever and showing more or less jaundice at the time of injection of this dose likewise survived, and in some jaundice rapidly disappeared in 24 hours. On the other hand, this quantity of serum failed to prevent a fatal outcome when given to animals in which defebrescence and increasing jaundice had set in. The nephritic symptoms, which had existed since 72 hours after inoculation, were rapidly increasing by this time.

0.0001 cc. of the serum prevented a fatal infection in three out of four guinea pigs when given within 24 hours. In one animal which

[3] Noguchi, H., *J. Exp. Med.*, 1920, xxxi, 159.

died from the typical infection there was a prolongation of the incubation period (6 days) and the duration of illness ($5\frac{1}{2}$ days). In two of the three surviving animals fever developed after 4 days, but they recovered without showing jaundice at any time. Injection of this quantity of serum 48 hours after inoculation, or later, had no effect upon the course of the disease, all animals so treated dying within 5 to $8\frac{1}{2}$ days with typical symptoms.

Seven guinea pigs which were near or in a state of collapse 6 days after the inoculation were given 1 cc. (three animals) and 2 cc. (four animals), but with one exception all died within $\frac{1}{2}$ to $2\frac{1}{2}$ days from the time of injection of the serum.

It may therefore be concluded that the anti-*icteroides* serum here employed is able, in a dose of 0.1 cc., to protect a guinea pig from a fatal infection against at least 5,000 minimum lethal doses of the Merida strain when administered during the incubation period or at any early stage of the disease (fever and beginning of jaundice), but that it has almost no effect upon the course of the disease if given at a later period when the animals are deeply yellow and the temperature has begun to go down. At or near collapse even 1 or 2 cc. failed to prevent death in the majority of animals. On the other hand, a quantity as minute as 0.0001 cc., when given within 24 hours, is able to protect the majority of animals from a fatal infection.

If man's degree of susceptibility to *Leptospira icteroides* is comparable to that of the guinea pig, it may reasonably be assumed that the injection of the anti-*icteroides* serum at an early period of the disease will have a beneficial effect similar to that observed in the treatment of the experimental infection in guinea pigs. On the basis of body weight a man of 80 kilos would require about 200 times the amount of serum needed to save a guinea pig of 400 gm.; that is, 20 cc. (0.1 cc. $\times$ 200 = 20 cc.). The mode of administration should be intravenous, and if necessary the injection should be repeated at short intervals (4 hours).

TABLE III.

Serotherapeutic Value of the Anti-icteroides Serum against 0.5 Cc. (5,000 Minimum Lethal Doses) of an Organ Emulsion, Merida Strain.

Length of time before serum injection.	Symptoms.	Amount of serum injected.			
		0.0001 cc.	0.1 cc.	1 cc.	2 cc.
hrs.					
1		No. 131. Incubation period 6 days. Died after $11\frac{1}{2}$ days.	No. 133. No infection.		
		No. 132. No infection.	" 134. " "		
24		" 135. Fever from 6th day on, but recovered.	" 137. " "		
		No. 136. Fever on 5th day, but recovered.	" 138. " "		
48		No. 139. Died after $6\frac{1}{2}$ days.	" 141. " "		
		No. 140. Died after 5 days.	" 142. " "		
72	Fever; no jaundice.	No. 143. Died after $7\frac{1}{2}$ days.	" 145. Survived without jaundice.		
		No. 144. Fever on 5th day. Died after $7\frac{1}{2}$ days.	No. 146. Survived without jaundice.		

days 4	Fever; jaundice.	No. 147. Died after 8½ days.	No. 149. Survived, but jaundice increased next day.		
		No. 148. Died after 6 days.	No. 150. Survived; jaundice disappeared next day.		
5	Temperature down; jaundice.	No. 151. Died after 6 days.	No. 153. Died after 8½ days.		
		No. 152. Died after 5½ days.	No. 154. Died after 7½ days.		
6	Near or in collapse.			No. 159. Died after 7½ days.	No. 155. Died after 8½ days.
				No. 160. Died after 8½ days.	No. 156. Died after 6½ days.
				No. 161. Died after 8 days.	No. 157. Died after 6½ days.
					No. 158. Recovered.

Pfeiffer Phenomenon with the Serums of Yellow Fever Convalescents in Merida.

Pfeiffer's phenomenon with *Leptospira icteroides* had been previously observed with a limited number of serums from yellow fever convalescents in Guayaquil[4] and was positive in about 85 per cent of the cases studied. It was desirable that this line of observation should be extended to yellow fever cases existing elsewhere, and we availed ourselves of the present opportunity with the Merida cases. Our first intention was to study as many specimens of serum from yellow fever convalescents in Merida as could be obtained, with as many strains of cultures of *icteroides* as possible. This plan could not be carried out, however, for two reasons; there were only a few convalescents accessible, and all the Guayaquil cultures brought down by us to Merida were lost within a few days after our arrival, and before our work had been started, by an accidental rise of temperature for 3 days to 56°C. of the thermostat in which the cultures had been placed for safety. Hence work on the Pfeiffer phenomenon was possible only after the isolation of *Leptospira icteroides* from Case 2.

There were two convalescents at the yellow fever hospital (Casa de Salud), Cases 2 and 3.[5] Both patients (soldiers) were infected in the same house in Merida, Case 3 preceding Case 2 by perhaps 2 weeks. The serum for the Pfeiffer test was drawn at the end of the 5th week of the disease in Case 3 and during the 3rd week in Case 2. Both specimens were still distinctly jaundiced. Through the courtesy of Dr. A. Lara we were given specimens of serum from two other convalescents who had had a typical attack of yellow fever in August, 1919; that is, about 5 to 6 months previously. One was a young woman, whose urine still contained albumin at that time, but who showed no bile pigment either in the urine or in the serum.

The following procedure was employed. 0.5 cc. of each specimen of serum was mixed with 1.5 cc. of a pure culture of the Merida strain of the leptospira and the whole injected into the peritoneal cavity of a guinea pig. The exudate was withdrawn from the peritoneal cavity after 30 minutes for dark-field examination. Table IV is a record of the results.

[4] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 9.

[5] Noguchi, H., *J. Exp. Med.*, 1920, xxxii, 601.

The serums from yellow fever convalescents in Merida gave uniformly a positive Pfeiffer reaction when tested with a culture of the leptospira isolated from Case 2. Although the guinea pig inoculated with the mixture of this culture and the serum from the same patient died of secondary infection too soon to permit any observation with regard to the power of the serum to protect the animal from a fatal infection, the animal which received the serum from Case 3 with this culture escaped the infection altogether. It may be supposed that this patient had been attacked by the same strain that later infected the other two soldiers (Case 1, fatal, and Case 2) living in the same

TABLE IV.

Pfeiffer Reaction with Serum from Convalescents and the Merida Strain.

Serum No.	Type of infection.	Length of time after onset of disease.	Pfeiffer reaction.	Remarks.
		wks.		
1 (Case 2).	Moderately severe.	3	Positive.	Died of intercurrent infection within 24 hrs.
2 (" 3).	Moderately severe.	5	"	No infection resulted.
		mos.		
3 (civilian).	Typical.	5	"	Died after 7 days with typical infection.
4 (").	"	6	"	Died after 7 days with typical infection.
No serum (control).			Negative.	Died after 9 days with typical infection.

house, hence such complete protection. On the other hand, Serums 3 and 4 were from two civilians who had recovered from yellow fever at least 5 months previous to the time when their blood was tested. These serums gave a positive Pfeiffer phenomenon but failed to protect the animals.

These four serums were brought back to The Rockefeller Institute to be tested with the Guayaquil strains of *Leptospira icteroides* and also with some of the strains of *Leptospira icterohæmorrhagiæ*. The serums from Cases 2 and 3 were accidentally lost during transportation. Moreover, during the journey the remaining two had to be

kept at a comparatively high temperature (heated steamer cabin and trains) for 15 days, and their activity must have suffered considerably. Nevertheless, tests were made on March 5; that is, 2 months from the time of collection. The amount available of Serum 3 was 0.75 cc. and that of Serum 4, 0.9 cc. Each specimen was divided into three equal parts and tested with two Guayaquil *icteroides* strains (Nos. 1 and 5) and with the American No. 1 strain of *icterohæmorrhagiæ*. The results obtained are presented in Table V.

TABLE V.

Pfeiffer Reaction with Serum from Convalescents Tested with Two Guayaquil Strains of Leptospira icteroides and One Strain of Leptospira icterohæmorrhagiæ.

Serum No.	*Leptospira icteroides.*		*Leptospira icterohæmorrhagiæ.*
	Guayaquil Strain 1.	Guayaquil Strain 5.	American Strain 1.
3	Positive.	Doubtful.	Negative.
4	Partial reaction.	Partial reaction.	"
No serum (control).	Negative.	Negative.	"

While no definite conclusions can be drawn from the results obtained with these old serums, it is possible to recognize an unmistakable specific reaction with the *icteroides* strains, particularly between Serum 3 and No. 1 of the Guayaquil *icteroides* strains. With Serum 4 there was not complete destruction of the *icteroides* organisms; but about one-half of them became paralyzed, and some were degenerated, although many active organisms were simultaneously present. There was no effect whatever upon American Strain 1 of *Leptospira icterohæmorrhagiæ*.

SUMMARY.

Identification of the leptospira isolated from a case of yellow fever in Merida was accomplished by means of an anti-*icteroides* immune serum prepared in a horse with several Guayaquil strains of *Leptospira icteroides*. The immune serum showed a protective action of high titer against the Merida strain, thus establishing its efficacy as a therapeutic agent against this strain. Polyvalent anti-*icteroides*

immune serum prepared in the horse or monovalent anti-*icteroides* immune serums prepared in the rabbit had a definite devitalizing action upon the Merida strain, while immune serums similarly prepared with strains of *icterohæmorrhagiæ* had no perceptible effect upon the Merida strain.

Serums from yellow fever convalescents in Merida gave a positive Pfeiffer reaction with the Merida strain of *Leptospira icteroides*. The reactions between the Guayaquil strains (Nos. 1 and 5) and two of these serums from convalescents varied from definitely positive to doubtful, owing probably to the diminution of active immune principles in the serums during the prolonged and unfavorable conditions of their transportation.

BIOLOGICAL AND PHYSICAL PROPERTIES OF THE HEMOTOXIN OF STREPTOCOCCI.

By PERCY D. MEADER, Ph.D., and GEORGE H. ROBINSON, Ph.D.

(*From the Department of Bacteriology of the School of Hygiene and Public Health, the Johns Hopkins University, Baltimore.*)

(Received for publication, June 17, 1920.)

The isolation of hemolytic streptococci from many different pathological lesions demonstrates their importance as pathogenic organisms. Various investigators point out two types of these organisms, one of which produces hemolysis only on blood agar, while the other produces also a hemolytic substance, or hemotoxin, in bouillon. Whether or not all laking of erythrocytes as exhibited on blood agar plates by different strains is the result of the same reaction is not clear. However, the ability to produce hemotoxin in bouillon seems to be a definite characteristic of certain strains of streptococci. Since hemolytic power is a biological function of most virulent streptococci, it is of interest to note the nature of the hemotoxin.

Frequent attempts have been made to separate hemotoxin from the organisms by filtration. The matter of filtration is of the first importance in showing whether the hemotoxin is in solution or is contained within the bacterial cells. Aronson (1902) states that hemotoxin can pass through a filter. Besredka (1901), using a Chamberland filter, noted a relation between the rate of filtration and the hemolytic strength of the filtrate and suggests that perhaps the active substance is retained in the pores of the filter. Ruediger (1903) recommends that the filter should be carefully selected, as too fine a filter removes the hemolytic property. Von Hellens (1913) and Nakayama (1919) were able to obtain hemolytic filtrates only with considerable loss of potency during the procedure. Maasen filters were used in their experiments. M'Leod (1911–12) and Braun (1912) were able to secure hemolytic filtrates by the use of Maasen filters. Lyall (1914) was not able to obtain hemolytic filtrates by the use of coarse Berkefeld filters.

Another method of separating the bacterial cells from the hemotoxin is by centrifugation. Levin (1903) reported that there was nearly 1,000 times as much hemotoxin in the sediment of a centrifuged tube of medium as in the filtrate of

the same fluid. A comparison of the sediment and the supernatant fluid after centrifugation would seem to have more value, however. Schlesinger (1903) concentrated the streptococci by centrifuging and found that hemotoxin was liberated by crushing the cells.

Little is recorded regarding the chemical nature of the hemotoxin. Ruediger (1903) states that it is composed of a toxophore and haptophore group loosely bound together. Von Hellens (1913) found hemotoxin soluble in ether and extracted it by this method. Von Lingelsheim (1912) reports that hemotoxin is of the nature of an enzyme. Lyall (1914) is of the opinion that it is not an enzyme because the hemolytic power of his cultures was destroyed by exposure to chloroform and toluene, reagents not destructive to enzymes.

The culture medium used by different workers for the production of hemotoxin varies widely. M'Leod (1911–12) found that the addition of 15 to 20 per cent of horse serum to bouillon produced the most powerful hemotoxin. Nakayama (1919) added 50 per cent or more horse serum to broth. Von Hellens (1913), on the other hand, found that the addition of horse serum in amounts of more than 40 per cent tended to decrease the amount of hemotoxin produced. Lyall (1914) used a calcium carbonate broth to which 16 per cent ascitic fluid was added.

Owing to the contradictory results reported in the literature a repetition of much of the work on this subject seemed necessary. The object of the work reported in this paper was to determine if possible something of the nature of the hemotoxin by means of a study of its filterability, the effect of adsorptive agents, the effect of centrifugation and shaking, and the basic substances from which it is produced.

In all the work a standard culture medium was used consisting of bouillon with 1 per cent of Difco peptone prepared according to the method of Huntoon (1918), to which were added 3 drops of normal horse serum per 5 cc. of the medium. The strains of streptococci used were obtained from cases of empyema and were supplied by the Army Medical School. Hemolytic tests were accomplished by adding to 0.5 cc. amounts of the proper dilutions of the test substance 0.5 cc. of 5 per cent washed rabbit corpuscles. The tubes were then incubated for 1 hour in the water bath at 37°C. The relative susceptibility of the corpuscles from different animal species was not determined. It was found that the hemotoxin of the streptococcus was not specific for red blood cells but had the power of destroying various types of muscular and glandular tissue.

Effect of Filtration and Adsorption on the Hemotoxin.

The effect of filtration on hemotoxin may be determined by a few simple experiments. It was found that there was considerable loss during passage of bouillon through Mandler filters. If the hemolytic substance is held in the pores of the filter, reversal of the filter and washing with the filtrate should restore the hemolytic titer. No hemotoxin was recovered by this method. The rate of filtration was observed to have considerable effect upon the amount of hemotoxin in the filtrate; *i.e.*, the more rapidly the bouillon was forced through the filter, the greater the amount of hemotoxin recovered. As a control on this experiment a broken filter was finely pulverized in a mortar and 2 gm. were added to each of two tubes containing 5 cc. of

TABLE I.

Treatment.	0.5 cc.	0.25 cc.	0.12 cc.	0.06 cc.	0.03 cc.	0.015 cc.
Untreated bouillon culture................	++	++	++	++	++	+
Rapid filtration..........................	++	++	+	−	−	−
Slow "	−	−	−	−	−	−
Reversal of the filter....................	−	−	−	−	−	−
Rapid adsorption..........................	++	++	++	++	−	−
30 min. "	−	−	−	−	−	−
Addition of calcium chloride..............	+	−	−	−	−	−

++ designates complete hemolysis; +, partial hemolysis; −, no hemolysis.

hemolytic bouillon culture. These were shaken to insure an even distribution of the pulverized material throughout the bouillon. One was immediately centrifuged and the other allowed to stand for 30 minutes before centrifugation. The first tube showed slight diminution of hemolytic titer, while the second showed no hemotoxin present in the liquid.

The addition of calcium chloride to bouillon causes a precipitate which may act as an adsorptive agent. Barium carbonate will also remove the greater part of the hemotoxin from bouillon.

From Table I the similarity in action between rapid filtration and adsorption can be observed. It is especially significant, since the same material was used in both processes. The failure to recover hemotoxin by reversal of the filter and passage of the filtrate back

through the filter indicates either the destruction of hemotoxin or its firm combination with the filter material. If infusion bouillon is passed through a diatomaceous filter before inoculation with hemolytic streptococci good growth results but the production of hemotoxin is greatly reduced, indicating that the basic substances from which the hemotoxin is derived are likewise adsorbed, at least in part, by filtration. The fact that the addition of insoluble compounds also removes hemotoxin from bouillon seems to confirm the part played by adsorption in the process of filtration.

Effect of Centrifugation and Shaking on the Hemotoxin.

If the hemotoxin is contained in or on the bacterial cells, concentration of the cells by centrifugation should increase the hemolytic titer. A tube of hemolytic bouillon was centrifuged at high speed until the supernatant liquid was clear (about 20 minutes). Another tube of bouillon fitted with a rubber stopper was placed on a wheel revolving about 30 times a minute and the bouillon was kept in constant agitation for 20 minutes. The top half of the liquid in the centrifuged tube was removed with a pipette; part of this was used for plating on blood agar in order to determine the number of cocci present and part used for hemolytic tests. The lower half was shaken in order to get an even distribution of the sedimented streptococci and was tested in a similar manner. As a control on the presence of streptococci some of the same bouillon was treated with the pulverized filter material for 20 minutes and centrifuged for 3 minutes. The results are shown in Table II.

The counts shown in this table are of necessity inaccurate, since we have no means of knowing whether one streptococcus or a chain of several members produced the colonies. They are, however, relative and give a fair ground for comparison. The hemotoxin has evidently disappeared from the supernatant fluid, although there are some organisms present. Even though the streptococci have been concentrated to twice the number per cubic centimeter the hemolytic titer is slightly less than that of the bouillon in the control tube which had not been centrifuged. The process of shaking also reduced the titer somewhat, although the bacterial count remained the same as in the

control tube. The culture treated with kieselguhr contains enough streptococci to show some hemolysis, provided the hemolytic substance is an integral part of the cell. If a hemolytic test is set up in a hollow culture slide and the process of hemolysis studied through the microscope it soon becomes evident that hemotoxin must be free in the medium and unassociated with the bacterial cells. Red blood corpuscles are seen to assume gradually a moth-eaten appearance and finally to disintegrate, although during the whole process no streptococci have been near them. In such a test as that shown in Table II there are present 100 erythrocytes to each streptococcus. Since these organisms are non-motile it is difficult to believe that each blood cell has come in direct contact with a streptococcus. Microscopic study gives convincing evidence that such is not the case. The results of these experiments seem to indicate that the hemotoxin is not associated with the bacterial cells and that there is a mechanical factor responsible for loss of titer during centrifugation.

TABLE II.

Treatment.	0.5 cc.	0.25 cc.	0.12 cc.	0.06 cc.	0.03 cc.	0.015 cc.	No. of colonies per cc.
Untreated	++	++	++	+	–	–	1,000,000
Centrifuged, top	–	–	–	–	–	–	50,000
" bottom	++	++	+	+	–	–	∞
Shaken	++	++	+	–	–	–	1,000,000
Kieselguhr	–	–	–	–	–	–	100,000

Relation of Hydrogen Ion Concentration to the Hemotoxin.

Certain concentrations of acid and alkali are suitable reagents for causing the laking of erythrocytes. The possibility that hemolysis may be due to this cause cannot be ignored. The presence in bouillon of carbohydrates from which acid might be produced tends, however, to decrease rather than increase the amount of hemotoxin. The only apparent effect of the initial hydrogen ion reaction of the medium was to inhibit growth when the reaction was strongly acid or alkaline. Hemotoxin was obtained from bouillon having a pH value at the time of testing for hemolysis of 7.2 to 5.3. Media containing pieces of marble gave maximum hemotoxin production. Hemolytic bouillon

diluted with an equal volume of isotonic sodium carbonate solution gave results identical with those obtained by diluting to the same extent with sodium chloride solution. From these results it seems impossible that the hemolytic action can be due to the presence of an acid radical.

Relation of the Constituents of the Medium to Hemotoxin Production.

The ideal method of attacking this phase of the problem would be to withdraw one substance after another from the culture medium and test each substance alone and also the remainder of the material as to its ability to support hemotoxin production. This is not possible in all cases on account of the complexity of the constituents of

TABLE III.

Bouillon.	Growth.	0.5 cc.	0.25 cc.	0.12 cc.	0.06 cc.	0.03 cc.	0.015 cc.
Undiluted......................	Good.	++	++	++	++	++	+
Diluted to 2 volumes..........	"	++	++	++	++	++	+
" " 4 "	"	++	++	++	++	++	+
" " 8 "	"	++	++	++	++	+	+
" " 16 "	Slight.	++	++	++	+	–	–
" " 32 "	"	++	++	+	+	–	–
" " 64 "	"	++	+	+	–	–	–
Horse serum + salt solution......	None.	–	–	–	–	–	–

the bouillon. We must constantly bear in mind the necessity of removing any bactericidal reagent from the medium and also of retaining the tonicity of the preparations at a point such that hemolysis of red blood cells will not follow from this cause.

It seemed probable that on occasions solutions would be added to the medium and it would be inconvenient and not expedient to evaporate the excess. The effect of diluting the bouillon with physiological salt solution was therefore investigated. As usual, 3 drops of horse serum were added to each tube after the dilutions were made.

From Table III it is evident that bouillon can be diluted to four times its volume before any effect on hemotoxin production is observed. Beyond this point the hemotoxin production is in inverse ratio to the dilution of the medium. In no case has the addition

of reagents approached four times the volume of the bouillon so any decrease in hemolytic titer is not due to the factor of dilution.

Relation of Phosphorus to Hemotoxin Production.—In Table I it was shown that the addition of calcium chloride to bouillon caused the formation of a precipitate. Upon analysis this proved to consist mainly of calcium phosphate, although other substances were brought down either mechanically or by adsorption. Phosphoproteins are not precipitated by this reagent. It was found, however, that streptococci during growth liberated phosphorus, presumably from the protein radicals. In order to obtain a phosphorus-free medium the following procedure was adopted. 0.6 cc. of 2.5 per cent calcium chloride solution was added to 5 cc. of bouillon and boiled. The precipitate was removed by centrifugation. The supernatant liquid

TABLE IV.

Bouillon.	Growth.	0.25 cc.	0.12 cc.	0.06 cc.	0.03 cc.	0.015 cc.
Bouillon B_1 with calcium chloride..........	Good.	++	++	++	++	+
" B_2 " " "	"	++	++	++	++	+
" B_3 " " "	"	–	–	–	–	–
" B_3 + ammonium monobasic phosphate..............................	"	++	+	+	–	–
Bouillon B_3 + phosphoric acid.............	"	++	++	+	–	–
" B_3 + potassium acid phosphate....	"	++	–	–	–	–

was enriched with 3 drops of normal horse serum, inoculated with streptococci, and incubated for 24 hours. Then the process was repeated until the medium was free from phosphorus. Two or three inoculations were generally necessary. Such a phosphorus-free bouillon reinforced by 3 drops of normal horse serum supported abundant growth of streptococci but allowed production of no hemotoxin.

The addition of known phosphorus compounds to a phosphorus-free bouillon was tried. In Table IV is a tabulation of a series of such experiments. Bouillon B_1 was heated with calcium chloride once; Bouillon B_2 was heated with calcium chloride, the precipitate removed, inoculated with streptococci, incubated, and precipitated a second time. No. B_3 was carried through the process of precipitation for a third time. When chemically tested this bouillon was shown to be free from phosphorus. The amounts of phosphorus compounds

added were 0.5 cc. of 2 per cent ammonium monobasic phosphate, 0.5 cc. of 2 per cent potassium acid phosphate, and 0.5 cc. of 1 per cent phosphoric acid. These solutions are approximately isotonic for red cells as they gave no hemolysis when added to a standard cell suspension.

The results of these experiments indicate that as long as the phosphoproteins are present in the medium the maximum amount of hemotoxin is produced. The addition of phosphorus compounds restores in part but not completely the hemotoxin-producing properties of the medium. The non-production of hemotoxin in phosphorus-free bouillon is not due to the effect of the lack of buffer action, as the reaction was unchanged by the growth of the streptococci and presented a range within which hemotoxin has been produced repeatedly.

Relation of Other Chemical Factors to Hemotoxin Production.—The preceding experiment and certain others suggested the possibility that there might be present in the medium two factors or chemical essentials, one of which is able to support growth but not hemolysin production, the other supplies the necessary substances for the elaboration of hemotoxin. Experiments were instituted to separate the chemical substances contained in infusion bouillon. Metaproteins, proteoses, and peptone were isolated from the bouillon according to the methods outlined by Hawk (1916). These substances were made up to the approximate volume of the bouillon from which they were taken by the addition of physiological salt solution, and the standard amount of normal horse serum was added. Fair growth of streptococci was invariably obtained but no hemotoxin was produced. Even the mixture of all these substances in the original proportion failed to support the production of hemotoxin. It seems evident that a variety of substances may support growth but not the production of hemotoxin.

Two reagents have been used for the fractionating of bouillon, calcium chloride and ether. When calcium chloride is added to broth a heavy precipitate is formed. This precipitate can be divided by ether extraction into a waxy substance, which is perhaps cerebrin, and calcium phosphate. Neither the whole precipitate nor either of the fractions, when washed free of bouillon, supported hemotoxin production. Bouillon after removal of the calcium precipitate suffers

no loss of its hemotoxin-producing qualities. Extraction of this fraction with ether does not affect the production of hemotoxin. When the whole bouillon is extracted with ether and then precipitated with calcium chloride the fractions containing the water-soluble matter are the only ones which give hemolysis. These preparations were brought as nearly as possible to the original volume and to them was added the usual amount of serum.

If the calcium precipitate previously referred to is returned to the bouillon no effect on hemotoxin production is observed. The substance separated from the bouillon by ether extraction, whether obtained from the whole bouillon or from the calcium precipitate, when returned to bouillon considerably reduces the yield of hemotoxin. Pure cholesterol when added to the bouillon does not influence hemotoxin production. Lecithin, however, has a pronounced inhibitory action. Thus the substance which is separated from the bouillon by means of ether acts in a manner similar to lecithin. The substances in bouillon which are soluble or insoluble in ether are not essential to the production of hemotoxin of the streptococcus.

Relation of Serum to Hemotoxin Production.—When bouillon prepared with Liebig's beef extract instead of meat infusion is planted with streptococci, fair growth is obtained but no hemotoxin is produced. By using this medium as a base, opportunity is offered for determining whether the source of the hemotoxin is in the infusion bouillon or in the blood serum. To one series of tubes containing 5 cc. of Liebig's extract bouillon graduated amounts of infusion bouillon were added; to the other the same amounts of serum were added. The results of these tests are shown in Table V.

From this table it can be seen that the infusion bouillon of itself adds but little to the production of hemotoxin, whereas serum increases the production in the ratio of its presence in the medium. There is, perhaps, the requisite amount of the growth factor in either Liebig's or infusion bouillon. The basic substances for hemotoxin production are present in only a slight degree in infusion bouillon but in considerable quantity in serum. It is evident that there are certain quantitative relations between the growth factor and the hemotoxin-producing factors to be observed in the production of the maximum amount of hemotoxin. With infusion bouillon 0.05 cc., or 3 drops, of

serum are sufficient for maximum hemotoxin production, whereas with Liebig's extract bouillon 2 cc. of serum are necessary to obtain the same result. The growth factor in infusion bouillon may be diluted to four volumes as shown in Table III and still produce the maximum amount of hemotoxin. Horse serum diluted with physiological salt solution will support little if any growth.

The isolation of the hemotoxin-producing substance from serum is as difficult as from bouillon. Serum was diluted to twice its volume with salt solution and heated in boiling water until the coagulable proteins were well separated. This preparation was centrifuged until the supernatant fluid, or serum water as we have designated it, was perfectly clear. When this serum water which amounted to 5

TABLE V.

Amount of infusion bouillon + 5 cc. of Liebig's extract bouillon.	Hemolysis produced.					Amount of horse serum + 5 cc. of Liebig's extract bouillon.	Hemolysis produced.				
	0.25 cc.	0.12 cc.	0.06 cc.	0.03 cc.	0.015 cc.		0.25 cc.	0.12 cc.	0.06 cc.	0.03 cc.	0.015 cc.
cc.						*cc.*					
0.05	+	−	−	−	−	0.05	−	−	−	−	−
0.2	++	+	−	−	−	0.2	+	−	−	−	−
0.6	++	+	−	−	−	0.6	++	++	+	+	−
1.0	++	+	−	−	−	1.0	++	++	++	+	−
2.0	++	+	−	−	−	2.0	++	++	++	++	++
2 cc. of infusion + 5 cc. of salt solution.	++	++	++	++	−	2 cc. of horse serum + 5 cc. of salt solution.	−	−	−	−	−

to 10 per cent of the original serum was made up to volume and added to bouillon, hemotoxin was produced in maximum quantity. Calcium chloride solution, when added to serum water, causes a precipitate, but the hemotoxin-producing factor is but slightly diminished in the supernatant fluid. The coagulable protein and the calcium precipitate of serum water do not support hemotoxin production and growth occurs to only a slight degree.

Hemotoxin-Producing Substance in Various Organs.—The occurrence of the hemotoxin-producing substance in different organs was investigated. Water extracts of brain, liver, and kidney were prepared by infusing the macerated tissues in an equal weight of water. The meat and precipitate were removed by centrifugation and the clear

extract was sterilized by boiling for 5 minutes. These preparations were added to Liebig's extract bouillon in amounts of 0.6 cc., a quantity in the case of serum sufficient to produce a fair amount of hemotoxin. The brain extract induced little or no hemotoxin production due, evidently, to the presence of a lipoid in the medium. The liver extract produced an unusual amount of growth but no hemotoxin. This result we considered as due to the selective action of the streptococcus for the glycogen in the liver extract. The kidney extract, however, when added to Liebig's bouillon produced hemotoxin equal in amount to that elaborated in the presence of serum or serum water. These results are similar to those obtained by Kligler (1919) in his study of the growth-producing substances in tissue.

The outstanding feature of these substances is their susceptibility to alkalinity and high temperatures. Kidney infusion was distributed in 5 cc. amounts into four test-tubes. To the first tube 1 cc. of normal potassium hydroxide was added; it was boiled for 5 minutes and then exactly neutralized with normal hydrochloric acid. To the second tube were added 1 cc. of normal potassium hydroxide and 1 cc. of normal hydrochloric acid and the whole was boiled at the same time as the first. To the third tube were added 2 cc. of sodium chloride solution and the entire contents were boiled as a control when the other tubes were boiled. The fourth tube was autoclaved for 30 minutes at 15 pounds pressure. The contents of the first tube when added to Liebig's extract bouillon in any amount failed to support hemotoxin production. The contents of the second and third tubes induced hemotoxin production equal to that obtained with serum. The autoclaved infusion acted in a manner comparable to that of infusion bouillon as shown in Table V.

At first one is tempted to designate the hemotoxin-producing substance by the term vitamine, as did Kligler (1919). It is doubtful whether the word as used by the physiological chemists can be justified in this application in bacteriology. The distinction between the growth factor and the factor producing hemotoxin is marked. A variety of substances may supply the needs for metabolism. Proteoses, peptone, probably some of the amino-acids, and sugars furnish the essentials for growth. The substance essential for hemotoxin production does not support the growth of the organisms. We believe that

hemotoxin is a compound synthesized from at least two basic substances; one of these is a phosphorus compound, the other a substance of unknown composition. The latter is present in fresh tissue and body fluids, is water-soluble, and is susceptible to certain destructive processes. The hemotoxin compound is easily destroyed by mechanical and physical methods and acts as an organic compound and not as an enzyme.

CONCLUSION.

The hemotoxin of streptococcus is a labile substance affected by centrifugation or shaking. It is adsorbed by various organic and inorganic substances. Hemotoxin is produced within a wide range of hydrogen ion concentrations. It is neither in nor on the bacterial cell but is free in the culture medium. It is probably not an enzyme. There are at least two substances which are essential to the medium for the elaboration of hemotoxin, one of which is phosphorus; the other is a substance of unknown composition. The unknown component is present in small quantities in unfiltered muscle infusion, but is more abundantly supplied by blood serum and kidney infusion. This substance is not an albumin, globulin, primary or secondary proteose, metaprotein, or peptone of the medium or enriching fluid. It is water-soluble, is destroyed by boiling in alkaline solution and by prolonged heating, and is removed to a considerable extent by passage through a diatomaceous filter.

BIBLIOGRAPHY.

Aronson, H., *Berl. klin. Woch.*, 1902, xxxix, 979.

Besredka, *Ann. Inst. Pasteur*, 1901, xv, 880.

Braun, H., *Centr. Bakt., 1te Abt., Orig.*, 1912, lxii, 383.

Hawk, P. B., Practical physiological chemistry, Philadelphia, 5th edition, 1916.

von Hellens, O., *Centr. Bakt., 1te Abt., Orig.*, 1913, lxviii, 602.

Huntoon, F. M., *J. Infect. Dis.*, 1918, xxiii, 169.

Kligler, I. J., *J. Exp. Med.*, 1919, xxx, 31.

Levin, E., *Nord. med. Arch.*, 1903, iii, 3 F., 1.

von Lingelsheim, W., in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1912, iv, 453.

Lyall, H. W., *J. Med. Research*, 1914, xxx, 487.

M'Leod, J. W., *J. Path. and Bact.*, 1911–12, xvi, 321.

Nakayama, Y., *J. Infect. Dis.*, 1919, xxv, 509.

Ruediger, G. F., *J. Am. Med. Assn.*, 1903, xli, 962.

Schlesinger, A., *Z. Hyg. u. Infectionskrankh.*, 1903, xliv, 428.

DIVISIONS OF THE SO CALLED FLEXNER GROUP OF DYSENTERY BACILLI.*

By WILBURT C. DAVISON M.D.

(*From the Department of Pediatrics and the Department of Pathology of the Johns Hopkins University, and the Harriet Lane Home of the Johns Hopkins Hospital, Baltimore.*)

(Received for publication, June 21, 1920.)

INTRODUCTION.

There is considerable confusion in regard to the existence of subdivisions of the mannitol-fermenting group of dysentery bacilli. Numerous attempts have been made to establish subdivisions on the basis of their biological and serological characteristics (1–8). My conclusions are derived from the fermentation and agglutination reactions of 77 cultures isolated from cases of clinical dysentery in children, 12 cultures from cases of dysentery in the American Expeditionary Forces, and stock cultures of the 5 English types of Flexner bacilli.[1]

By the Flexner group I refer to Gram-negative non-motile bacilli, isolated from the stools of cases of clinical dysentery and agglutinated by the patient's serum. They produce a small amount of indole, do not liquefy gelatin, fail to ferment lactose, and produce acid and no gas in dextrose and mannitol. These are the only constant cultural characteristics. It would seem advisable to apply one name, *Bacillus dysenteriæ* Flexner, to this whole group. These mannitol-fermenting dysentery bacilli have been subdivided in two ways, first by fermentation tests with maltose, saccharose, dulcitol, and rhamnose, and secondly by agglutination tests with the sera of immunized rabbits.

* Read before the 7th annual meeting of the American Association of Immunologists, New York, N. Y., April 1, 1920.

[1] For the technique employed and a description of the cases of dysentery in children see Davison, W. C., Bacillary dysentery in children, *Bull. Johns Hopkins Hosp.*, 1920, xxxi, 225.

Divisions Based on Carbohydrate Fermentation.

In this country it has been customary to refer to the Flexner (9), Harris, Hiss-Russell (10), and Strong (11) divisions on the basis of differences in the fermentation of maltose and saccharose. In my series, by utilizing the differences of fermentation of dulcitol and rhamnose, three additional divisions were found in a few cases (Table I).

TABLE I.

Types of Fermentation of Flexner Dysentery Bacilli.

Fermentation type.	Name.	Motility.	Fermentation of test substances.									Per cent of cases.
			Indole.	Gelatin.	Lactose.	Dextrose.	Mannitol.	Dulcitol.	Rhamnose.	Maltose.	Saccharose.	
1	Hiss-Russell..........	0	+	0	0	+	+	0	0	0	0	7
2	Flexner, English Y....	0	+	0	0	+	+	0	0	+6	0	41
3	Strong...............	0	+	0	0	+	+	0	0	0	+13	14
4	Harris, English V, X..	0	+	0	0	+	+	0	0	+	+15	24
5	English Z............	0	+	0	0	+	+	0	+12	+4	+12	7
6	*B. alkalescens*.........	0	+	0	0	+	+	+5	0	+	0	3
7		0	+	0	0	+	+	0	+	0	+6	3

If late fermentation occurred, the day of its appearance is designated by the number placed after the + sign. Otherwise + designates acid (no gas) production on the 1st day and indole production on the 7th day. 0 denotes no fermentation (incubation continued 21 days).

Fermentation Type 7 was also isolated from a diarrhea case which was not clinically dysentery.

Other workers using still different carbohydrates have reported additional divisions (6, 12–14). In fact, if fermentation is accepted as a criterion for differentiation, still further members will probably be added in the future as new carbohydrates are used for fermentation tests, and even more confusion will result.

It has been demonstrated (7, 15) that dysentery cultures may lose or gain ability to ferment maltose, saccharose, and also dulcitol (4) and that distinctions made on this basis are inconstant and unreliable. In the same stool I have isolated cultures that fermented maltose and others that failed to do so (Case 53, Table II). In another

TABLE II.

Discrepancies between Fermentation and Agglutination Divisions of Flexner Dysentery Bacilli in Children.

Case No. (Culture No.).	Agglutination type of stool culture. Type sera. V	W	X	Y	Z	Summary. Fermentation type.	Agglutination type.
53*† (L156), 6* (69, 70, 73)	0	+125	0	±200	0	1	W, Y
6* (72, 74)	±200	0	0	0	0	1	V
39*(L61), 40* (L56, L57, L58)	0	0	0	±200	0	2	Y
39* (L51), 40* (L59), 46* (L82), 53*† (L154), 80* (L11), 10* (161, 174, 175A)	0	0	0	0	0	2	0
46* (L83, L84), 52 (L166, L167), 53*† (L155), 54 (L159)	0	+250	+250	±500	0	2	W, X, Y
80* (L9)	±125	±250	0	±300	0	2	V, W, Y
80* (L10, L12), 81 (L30, L32, L33), 8*† (107, 113, 114, 115, 116), 10* (156, 159, 162, 164, 186, 157)	0	±250	0	±250	0	2	W, Y
9 (153, 154)	+1,250	0	0	0	±500	2	V, Z
Y stock.	+125	0	+250	+625	±100	2	V, X, Y, Z
58 (L174, L175)	0	0	0	0	0	3	0
77 (L186, L187)	0	0	±1,250	+200	±1,250	3	X, Y, Z
5 (67)	+500	0	±200	0	±500	3	V, X, Z
V stock.	+1,250	0	0	+250	0	4	V, Y
X "	0	0	+250	0	0	4	X
41* (L65)	0	0	0	0	0	4	0
41* (L66), 45* (L75, L77), 63* (L114)	0	0	+500	0	+250	4	X, Z
41* (L67)	0	0	0	0	±200	4	Z
41* (L68, L69), 13*† (262, 268)	±200	0	+200	0	+250	4	V, X, Z
45* (L76)	0	+100	+250	±200	0	4	W, X, Y
63* (L115, L116), 78 (L188)	0	0	+500	+200	+150	4	X, Y, Z
79*† (L2, L5), 13*† (261, 263, 266, 270, 271, 274)	±1,000	0	0	0	±250	4	V, Z
13*† (260)	+1,250	0	0	±200	±250	4	V, Y, Z
13*† (267), 79*† (L4)	+500	0	0	0	±500	5	V, Z
Z stock.	0	0	+625	0	+625	5	X, Z
8*† (108)	0	0	0	0	0	6	0
69 (L111, L112), 39 (152)	0	0	0	0	0	7	0

Cases marked with an asterisk (*) had more than one serological type and those marked with a dagger (†) had more than one fermentation type of Flexner dysentery bacillus in the stools. Culture numbers preceded by L, *i.e.* L156, were from cases in Baltimore, the others were from Birmingham, Alabama. + and ± indicate complete and partial agglutination; the numbers following these signs refer to the dilutions (end-point) of the serum. Case 69 was a diarrhea which was not clinically dysentery.

dysentery specimen I have found organisms that differed in dulcitol fermentation (Case 8, Table II). In other stools I have isolated dysentery bacilli that differed in rhamnose fermentation (Cases 13 and 79, Table II).

It is, of course, possible that these represent mixed infections, but this is impossible to prove, inasmuch as the fermentation differences in maltose, saccharose, dulcitol, and rhamnose, as will be pointed out later, do not parallel the serological findings.

Divisions Based on Agglutination with Monovalent Rabbit Sera.

Murray (1) in England has discarded the fermentation divisions of the Flexner group and has found that thirty-four cultures isolated from cases of dysentery in different parts of the world fell into five divisions on the basis of their agglutination reactions with monovalent rabbit sera. These he designates as the V, W, X, Y, and Z divisions of the Flexner group.

As Murray emphasizes, these divisions are not definite but probably indicate that one or more antigens predominate in a given strain. With the English cultures and sera, by means of Dreyer's (16, 17) technique with formolized cultures, I have found that although each serum usually agglutinates its own organism to higher titer than other types (Table III), yet the V serum will also agglutinate the Y culture, the X serum will agglutinate Y and Z, the Y serum will agglutinate the V culture, the Z serum will agglutinate the Y culture, while the W serum, which is of low titer, appears to be specific. Murray states that absorption tests do not make these serological divisions more distinct. It may perhaps be found that agglutination with the sera of animals other than the rabbit may give more clear-cut results, as has been demonstrated (18) with sheep serum for streptococci.

With the cultures which I have isolated there is even more cross-agglutination (Tables II and IV). Some cultures that are agglutinated to end-titer by V serum react to quarter titer with Z. W, X, and Y sera frequently agglutinate the same cultures. With other cultures further combinations were found.

Among 62 cultures from children, of which many reacted with more than one serum (Table II), there were 21 positive agglutination

reactions with V sera, 28 with W, 21 with X, 38 with Y, and 28 with Z, while 15 cultures failed to agglutinate with any of these sera. It is probable that at least 2 of these 15 inagglutinable cultures (Nos. 174 and 175, Tables II and IV) represented a type differing from

TABLE III.

Cross-Agglutination of the Five English Type Sera.

Serum.	Culture.	Agglutination titer.					
		1:100	1:125	1:250	1:625	1:1,250	1:2,500
V	V	+	+	+	+	+	0
	W	0					
	X	0					
	Y	+	+	0			
	Z	0					
W	V	0					
	W	+	+	+	0		
	X	0					
	Y	0					
	Z	0					
X	V	0					
	W	0					
	X	+	+	+	0		
	Y	+	+	+	0		
	Z	+	+	+	+	0	
Y	V	+	+	+	0		
	W	0					
	X	0					
	Y	+	+	+	+	0	
	Z	0					
Z	V	0					
	W	0					
	X	0					
	Y	+	0				
	Z	+	+	+	+	0	

Murray's five, for the patient's serum (Case 58, Table IV) had no agglutinins for the V, W, X, Y, and Z stock cultures. Further artificial cultivation might have rendered the others agglutinable, but the destruction of the laboratory and cultures by fire prevented it.

TABLE IV.

Correlation of Patient's Serum Reactions and Types of Stool Organisms.

Case No.	Length of time after onset.	Patient's serum reactions. Type cultures.					No. of culture from stool.	Agglutination type of stool culture. Type sera.					Summary.		
		V	W	X	Y	Z		V	W	X	Y	Z	Type of patient's serum reaction.	Agglutination type of stool culture.	Fermentation type of stool culture.
	days														
39	200	0	0	0	±50	0	61	0	0	0	±200	0	Y	Y	2
							51	0	0	0	0	0		0	2
40	205	±200	±200	0	±200	0	56, 57, 58	0	0	0	±200	0	V, W, Y	Y	2
							59	0	0	0	0	0		0	2
46	21	+250	+1,000	+100	+250	±20	83, 84	0	+100	+250	+500	0	V, W, X, Y, Z	W, X, Y	2
							82	0	0	0	0	0		0	2
54	14	+100	0	0	+100	0	159	0	+250	±200	±500	0	V, Y	W, X, Y	2
80	19	+20	±20	0	+200	±20	9	±125	±250	0	±300	0	V, W, Y, Z	V, W, Y	2
	38	+100	±50	0	±200	±20	10, 12	0	±250	0	±250	0		W, Y	2
							11	0	0	0	0	0		0	2
58	6	0	0	0	0	0	174, 175	0	0	0	0	0	0	0	3
77	26	±50	0	0	±50	0	186	0	0	±1,250	+200	±1,250	V, Y	X, Y, Z	3
	76	+20	0	0	0	0	187						V		

41	184	0	±20	0	0	0	66	0	0	+500	0	+250	W	X, Z	4
							68, 69	±200	0	+250	0	+500		V, X, Z	4
							67	0	0	0	0	±150		Z	4
							65	0	0	0	0	0		0	4
63	68	0	0	+20	0	0	115, 116	0	0	+500	+200	+150	X	X, Y, Z	4
							114	0	0	+500	0	±150		X, Z	4
78	13	±20	±20	0	+20	0	188	0	0	+1,250	+500	+500	V, W, Y	X, Y, Z	4
79	17	+200	0	0	+100	+20	2, 5	±1,000	0	0	0	±250	V, Y, Z	V, Z	4
	21	+100	0	0	+100	+50	4	±1,000	0	0	0	±250		V, Z	5
69		0	0	0	0	0	111, 112	0	0	0	0	0	0	0	7

+ and ± indicate complete and partial agglutination; the numbers following these signs refer to the dilutions (end-point) of the serum. 0 indicates no agglutination with the patient's sera at 1:20, and with the diagnostic type sera at 1:150.

In other words, although a distinct division was not obtained, agglutination reactions with the five English type sera and these mannitol-fermenting dysentery bacilli lead to the assumption that different antigens exist and that there are more than five which must be recognized.

Discrepancies between Fermentative and Serological Divisions.

There is no correlation between the divisions by fermentation tests and the serological results. Organisms of the same fermentation reactions were agglutinated by different sera. As an example (Table II), among the group that ferment maltose and saccharose, usually called the Flexner-Harris division (Fermentation Type 4), some cultures were agglutinated only by the Z serum, some by the X, Y, and Z, some by the V and Z, some by the X and Z, some by the W, X, and Y, and still others by the V, X, and Z sera, etc. Conversely, organisms agglutinated by the same sera may have different fermentation reactions. It was also noted that among organisms from the same stool with the same fermentation reactions, some were agglutinated by one or more of the sera while others were inagglutinable.

With the English stock cultures (Tables I and III) the same lack of correlation exists; for instance, the Y and X cultures differ in saccharose fermentation, yet both are agglutinated by the X serum.

Levine (3) has laid emphasis on the fact that the Z culture is the only one to ferment rhamnose, yet it is agglutinated by the X as well as the Z serum. Two rhamnose fermenters isolated from dysentery cases were agglutinated by both V and Z sera. Three rhamnose fermenters, isolated from cases of diarrhea which were not clinical dysentery, were not agglutinated by Z or any of the other sera. Furthermore, the Z serum agglutinated many strains that did not ferment rhamnose. From one stool (Case 79, Table IV) one organism that fermented rhamnose and two that failed to ferment it were agglutinated by V and Z sera.

Dulcitol-fermenting dysentery bacilli from one case were agglutinated by the Y serum and those from another were inagglutinable. This division has been named *Bacillus alkalescens* by Andrewes (2) who regards it as non-pathogenic.

Correlation of Agglutination Reactions of the Patient's Serum and Fermentation and Serological Types of His Stool Organisms.

A partial correspondence exists between the serological type of the organisms isolated from the patient's stool and the agglutination reactions of his serum.

In all except one case (Table IV), if the patient's serum agglutinated any of the five stock antigens, the organisms isolated from the stool reacted with one or more of the sera of the same types. In many cases, however, the stool organisms were not agglutinated by all the types for which the patient had agglutinins. As an example, one patient's serum (Case 40, Table IV) had agglutinins for Types V, W, and Y, while the dysentery bacillus isolated from his stool reacted with only the Type Y serum.[2]

The reverse of this was also true; namely, that the patient's serum would not have agglutinins for all the types which were represented by his stool organisms; for instance, the organisms isolated from a patient's stool (Case 63, Table IV) were agglutinated by the X, Y, and Z sera, while his serum had agglutinins for only the X antigen. These illustrations indicate an overlapping of the antigenic content of these strains.

In the only instance in which there was no correspondence (Case 41, Table IV), the serum of the patient agglutinated the W culture while the organisms from the stool reacted with the V, X, and Z sera.

In a few cases in which tests were repeated after various intervals, if agglutinins persisted, they were of the same types that had been present in previous tests.

CONCLUSIONS.

From these data it is seen that ill defined divisions of the so called Flexner group exist. The divisions do not appear to be sufficiently distinct to warrant the use of separate names. To avoid confusion all mannitol-fermenting dysentery bacilli should be called *Bacillus*

[2] It might be argued that had more colonies been studied in the stool culture, Types V and W might have been found; but this would not explain the instances in which the patient's serum did not have agglutinins for all the types found in his stool.

dysenteriæ Flexner and the subdivision noted. There are two methods for this division, one by the fermentation of carbohydrates, the other by agglutination with monovalent rabbit sera. These do not coincide and one or the other and not both must be adopted. Inasmuch as Murray studied organisms from widely distributed sources, it would seem preferable to adopt his serological classification and to add to it the types that fail to be agglutinated by his V, W, X, Y, and Z sera, as this method is simpler and more rapid. The results of the agglutination reactions of the patient's serum may be expressed in the same terms as the serological typing of the organism from his stool. Fermentation is less constant and gives rise to more divisions than there are carbohydrates.

TABLE V.

Agglutinin Content of Polyvalent Therapeutic Serum.

Serum.	Culture.	Agglutination titer.							
		1:100	1:125	1:250	1:625	1:1,250	1:2,500	1:6,250	1:12,500
Polyvalent therapeutic serum.	V	+	+	+	+	+	+	0	
	W	+	+	+	+	+	+	+	0
	X	+	+	0					
	Y	+	+	+	+	+	+	+	0
	Z	+	+	+	+	+	0		

The serological reactions of these type sera, as Murray points out, show cross-agglutination to a greater or less extent, but they indicate that there are five antigens, V, W, X, Y, and Z and probably others, one or more of which predominate in a given strain. Polyvalent diagnostic and therapeutic sera are practically worthless unless they include antibodies for the more common of these types.

The diagnostic importance of recognizing that there are five or more antigens in this group is seen from the fact that the sera of some patients react with one, others with another, and that unless several antigens are used, some positive tests may be missed.

The therapeutic importance is emphasized by the fact that probably the best polyvalent therapeutic serum at present available has a very low titer for the X antigen, although that type was found in many of these cases (Table V).

BIBLIOGRAPHY.

1. Murray, E. G. D., An attempt at classification of *Bacillus dysenteriæ* based upon an examination of the agglutinating properties of fifty-three strains, *J. Roy. Army Med. Corps*, 1918, xxxi, 257, 353.
2. Andrewes, F. W., Dysentery bacilli: the differentiation of the true dysentery bacilli from allied species, *Lancet*, 1918, i, 560.
3. Levine, M., Dysentery and allied bacilli, *J. Infect. Dis.*, 1920, xxvii, 31.
4. Dudgeon, L. S., et al., Studies of bacillary dysentery occurring in the British forces in Macedonia, *Med. Research Com., Nat. Health Insurance, Special Rep. Series, No. 40*, 1919.
5. Dobell, C., Gettings, H. S., Jepps, M. W., and Stephens, J. B., A study of 1,300 convalescent cases of dysentery from home hospitals; with special reference to the incidence and treatment of amœbic dysentery carriers, *Med. Research Com., Nat. Health Insurance, Special Rep. Series, No. 15*, 1918.
6. Torrey, J. C., A comparative study of dysentery and dysentery-like organisms. Part I, Bacilli producing a typical reaction in litmus milk, *J. Exp. Med.*, 1905, vii, 365; Part II, Dysentery-like organisms which produce an atypical reaction in litmus milk and which should probably be included within the group of pseudo-dysentery bacilli, 385.
7. Sonne, C., Ueber die Bakteriologie der giftarmen Dysenteriebacillen (Paradysenteriebacillen), *Centr. Bakt., 1te Abt., Orig.*, 1915, lxxv, 408. Hehewerth, F. H., Über Dysenterie-Bacillen und ihre Einteilung in Grüppen, *Centr. Bakt., 1te Abt., Orig.*, 1916, lxviii, 3. Thjøtta, T., On the bacteriology of dysentery in Norway, *J. Bact.*, 1919, iv, 355. Gettings, H. S., and Douglas, S. R., An investigation of the Flexner-Y group of dysentery bacilli, *Med. Research Com., Nat. Health Insurance, Special Rep. Series, No. 30*, 1918.
8. Kruse, W., Über die Ruhr als Volkskrankheit und ihren Erreger, *Deutsch. med. Woch.*, 1900, xxvi, 637; Weitere Untersuchungen über die Ruhr und die Ruhrbazillen, 1901, xxvii, 370, 386; Die Blutserumtherapie bei der Dysenterie, 1903, xxix, 49; Zur Geschichte der Ruhrforschung und über Variabilität der Bakterien, 201; Neue Untersuchungen über die Ruhr, 1907, xxxiii, 292, 338; Der jetzige Stand der Dysenteriefrage, *Deutsch. Aerzte-Ztg.*, 1902, i, 25; Die Gesundheitsverhältnisse der Aerzte, Geistlichen und Oberlehrer im Vergleich mit denen anderer Berufe, *Centr. allg. Gsndhtspflg.*, 1900, xix, 229. Dienes, L., Beobactungen über das serologische Verhalten der giftarmen Dysenteriestämme, *Z. Immunitätsforsch., Orig.*, 1919, xxviii, 456. Douglas, S. R., Colebrook, L., and Morgan, W. P., Report upon a series of cases of dysentery received from the Mediterranean Expeditionary Forces, *Med. Research Com., Nat. Health Insurance, Special Rep. Series, No. 6*, 1917. Glynn, E., Berridge, E. M., Foley, V., Price, M., and Robinson, A. L., A report upon 2,360

enteritis "convalescents" received at Liverpool from various expeditionary forces, *Med. Research Com., Nat. Health Insurance, Special Rep. Series, No. 7*, 1918. Park, W. H., personal communication. Park, W. H., Dunham, E. K., Collins, K. R., and Payne, W. A., A clinical and bacteriological study of a number of outbreaks of disease due to the dysentery bacillus of Shiga, *Bull. Med. Sc.*, 1902, ii, 187. Park, W. H., Collins, K. R., and Goodwin, M. E., The dysentery bacillus group and the varieties which should be included in it, *J. Med. Research*, 1904, xi, 553. Park, W. H., and Carey, H. W., The presence of the Shiga variety of dysentery bacilli in an extensive epidemic of dysentery, *J. Med. Research*, 1903, ix, 180. Park, W. H., On the interpretation of reactions of agglutination among the bacilli of dysentery, *Med. Rec.*, 1903, lxiii, 358. Martini, E., and Lentz, O., Ueber die Differenzirung der Ruhrbacillen mittels der Agglutination, *Z. Hyg. u. Infectionskrankh.*, 1902, xli, 540. A study of the serological races of the Flexner group of dysentery bacilli, *Med. Research Com., Nat. Health Insurance, Special Rep. Series, No. 42*, 1919. Winslow, C.-E. A., Kligler, I. J., and Rothberg, W., Studies on the classification of the colon-typhoid group of bacteria with special reference to their fermentative basis, *J. Bact.*, 1919, iv, 429.

9. Flexner, S., On the etiology of tropical dysentery, *Philadelphia Med. J.*, 1900, vi, 414; The etiology of tropical dysentery, *Centr. Bakt., 1te Abt.*, 1900, xxviii, 625; A comparative study of dysenteric bacilli, 1901, xxx, 449; A comparative study of dysentery bacilli, *Brit. Med. J.*, 1901, ii, 786; On the etiology of tropical dysentery, *Bull. Johns Hopkins Hosp.*, 1900, xi, 231. Flexner, S., and Barker, L. F., Report of a special commission sent to the Philippines by the Johns Hopkins University to investigate the prevalent diseases of the islands, *Johns Hopkins Univ. Circ.*, 1900, xix, 13.
10. Hiss, P. H., Jr., and Russell, F. F., A study of a bacillus resembling the bacillus of Shiga, from a case of fatal diarrhœa in a child, *Med. News*, 1903, lxxxii, 289; and *Med. Rec.*, 1903, lxiii, 357; On fermentative and agglutination characters of bacilli of the "dysentery group," *J. Med. Research*, 1904–05, xiii, 1.
11. Strong, R. P., and Musgrave, W. E., Report on the etiology of the dysenteries of Manila, *Rep. Surg.-Gen. Army*, 1900, 251; The bacillus of Philippine dysentery, *J. Am. Med. Assn.*, 1900, xxxv, 498. Strong, R. P., Bacillary dysentery, in Forscheimer, Therapeutics of internal diseases, New York and London, 1914, v, 253.
12. Ruffer, M. A., and Willmore, J. G., On the etiology of dysentery, *Brit. Med. J.*, 1909, ii, 862; The serum treatment of dysentery with notes on the bacteriological diagnosis of dysentery, 1910, ii, 1519.
13. Eyre, J. W. H., Asylum dysentery in relation to *B. dysenteriæ*, *Brit. Med. J.*, 1904, i, 1002.

14. Castellani, A., La malattia del sonno, *Riv. crit. clin. med.*, 1903, iv, 652; On hæmolysins produced by certain bacteria, *Lancet*, 1902, i, 440; Some experiments on the precipitins, 1827; Ueber das Verhältniss der Agglutinine zu den Schutzköpern, *Z. Hyg. u. Infectionskrankh.*, 1901, xxxvii, 381; Some researches on the etiology of dysentery in Ceylon, *J. Hyg.*, 1904, iv, 495. Castellani, A., and Chalmers, A. J., Manual of tropical medicine, London and New York, 2nd edition, 1913, 1380.

15. Aveline, H. T. S., Boycott, A. E., and MacDonald, W. F., *Bacillus dysenteriæ* of Flexner in relation to asylum dysentery, *J. Hyg.*, 1908, viii, 309. Barber, M. A., The variability of certain strains of dysentery bacilli as studied by the single cell method, *Philippine J. Sc., Sect. B*, 1913, viii, 539. Martin, C. J., and Williams, F. E., Types of dysentery bacilli isolated at No. 3 Australian General Hospital, Cairo, March–August, 1916, with observations on the variability of the mannite fermenting groups, *J. Hyg.*, 1917–18, xvi, 257.

16. Dreyer, G., Widal's reaction with sterilised cultures, *J. Path. and Bact.*, 1909, xiii, 331; On Anvendelse af dræbt kultur til Widal-Reaktion, *Hospitalstid.*, 1906, 4. R., xiv, 532; Proceedings of the British Medical Association, Section of Pathology, Discussion on immunity, *Brit. Med. J.*, 1904, ii, 564.

17. Davison, W. C., The diagnosis of enteric fever (typhoid and paratyphoid A and B) by agglutination tests, *J. Am. Med. Assn.*, 1916, lxvi, 1297; Report on prophylactic vaccination with *B. typhosus*, *B. paratyphosus* A, and *B. paratyphosus* B, *J. Lab. and Clin. Med.*, 1916–17, ii, 607.

18. Dochez, A. R., Avery, O. T., and Lancefield, R. C., Studies on the biology of streptococcus. I. Antigenic relationships between strains of *Streptococcus hæmolyticus*, *J. Exp. Med.*, 1919, xxx, 179.

BIOLOGICAL STUDY OF THE HEMOPHILIC BACILLI.

By ERNEST G. STILLMAN, M.D., and JANET M. BOURN.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 10, 1920.)

In 1892 Pfeiffer[1] described a small Gram-negative bacillus which he associated with the disease influenza. Ultimately this bacillus was quite widely considered as the cause of influenza. The bacillus of Pfeiffer, or *B. influenzæ*, as it was eventually named, is a very small, non-motile, non-sporulating, faintly staining organism with rounded ends. It is irregular in form with a tendency to show bipolar staining. Coccoid forms are also frequently seen, and occasionally small chains of bacilli occur. Pfeiffer recovered the bacillus by smearing pus from bronchial secretions over serum agar, but subcultures failed to grow. He finally discovered that it was the hemoglobin in the pus which enabled the bacillus to grow. Thus this organism came to be known as a hemophilic bacillus. Since Pfeiffer's discovery, various Gram-negative hemophilic bacilli have been described, such as the pseudoinfluenza bacillus, Jochmann's bacillus, Muller's "trachoma bacillus," the Koch-Weeks bacillus, etc. The more recent studies of Wollstein[2] would seem to indicate that while there are minor morphological and cultural differences between these hemophilic bacilli, the distinctions are so slight that the various hemophilic bacilli should be considered identical with *B. influenzæ* or, at most, as varieties of the same species.

The epidemic of influenza in 1918 called to attention the lack of knowledge concerning the biology and epidemiology of the hemophilic bacilli despite the large amount of work done in connection with influenza. In a study of the occurrence of *B. influenzæ* in normal mouths, Pritchett and Stillman[3] described a hemophilic bacillus strikingly similar to, but distinguished from *B. influenzæ* by its ability to hemolyze blood. The colony of this organism cannot be differentiated from that of *B. influenzæ* on oleate agar or chocolate medium, and morphologically the differences are so slight that they cannot be relied upon. As a rule, the so called Bacillus X is slightly larger and coarser than *B. influenzæ* in stained films. The easiest method of differentiation is by growth on blood agar, upon which Bacillus X shows varying degrees of hemolysis. The majority of the strains of this organism actively hemolyze the surface of a blood agar plate and also hemolyze blood broth. An occasional strain is encountered, however,

---

[1] Pfeiffer, R., *Z. Hyg. u. Infectionskrankh.*, 1892, xiii, 357.

[2] Wollstein, M., *J. Exp. Med.*, 1915, xxii, 445.

[3] Pritchett, I. W., and Stillman, E. G., *J. Exp. Med.*, 1919, xxix, 259.

whose hemolytic powers are not well developed. If good growth is obtained in plain broth enriched with 2 per cent blood extract, hemolysis may be demonstrated by the use of a 5 per cent solution of washed rabbit blood corpuscles. 1.5 cc. of an 18 hour broth culture added to 0.5 cc. of the washed blood cells and placed in a water bath at 37.5°C. for 1 hour usually cause complete hemolysis. Jordan[4] in 1919 called attention to the fact that certain strains of *B. influenzæ* produced indole. Wadsworth and Wheeler,[5] in their work with *B. influenzæ*, note the production of gas, and also the fermentation of monosaccharides by some strains. Rivers[6] reported that certain strains of *B. influenzæ* produced indole and amylase and could reduce nitrates to nitrites.

In 1906 Bordet and Gengou[7] succeeded in cultivating a small, ovoid, Gram-negative bacillus which they had observed in the sputum of children suffering from pertussis. This bacillus, although very similar morphologically to *B. influenzæ*, is less pleomorphic, slightly larger, and generally appears more ovoid. After frequent subcultures *B. pertussis* grows on ordinary media without the presence of hemoglobin. It grows much more slowly during the first 24 hours of incubation than *B. influenzæ*. On blood agar a fine film of growth is barely visible at the end of 24 hours, while at the end of 48 hours a heavy grayish growth has developed which is very different from the appearance of *B. influenzæ* grown under the same conditions. Ferry and Noble[8] have stated that there is an apparent close relation between *B. pertussis* and *B. bronchisepticus*, although the latter grows luxuriantly on ordinary media and is motile. The bacillus of rabbit septicemia, while not hemophilic, presents a striking morphological likeness to *B. influenzæ*. Because of the morphological resemblance of these various bacilli to *B. influenzæ* a few strains are included for comparison in the present study of the hemophilic bacilli.

As the hemophilic bacilli are delicate organisms which do not grow readily on artificial media special attention must be paid to minute details of technique. This fact is well exemplified by the difficulty with which *Bacillus influenzæ* was cultivated before the use of special media such as oleate agar and chocolate agar and probably in large part accounts for our lack of knowledge of the biology of this delicate organism. Freshly prepared medium adjusted to the optimum hydrogen ion concentration, pH 7.3 to 7.5, is essential for growth.

[4] Jordan, E. O., *J. Am. Med. Assn.*, 1919, lxxii, 1542.

[5] Wadsworth and Wheeler, in Park, W. H., and Williams, A. W., Pathogenic microörganisms, Philadelphia and New York, 7th edition, 1920, 457.

[6] Rivers, T. M., *Bull. Johns Hopkins Hosp.*, 1920, xxxi, 50.

[7] Bordet, J., and Gengou, O., *Ann. Inst. Pasteur*, 1906, xx, 731.

[8] Ferry, N. S., and Noble, A., *J. Bact.*, 1918, iii, 193.

EXPERIMENTAL.

In the present paper are reported the data obtained during an investigation of the hemophilic bacilli recovered from the throats and sputum of patients suffering from acute influenza and lobar pneumonia, and from the throats and saliva of healthy individuals. The study includes the facts obtained concerning (1) final hydrogen ion concentration, (2) sugar fermentation, (3) indole production, (4) nitrate reduction, and (5) gas production. In addition to the strictly hemophilic organisms, *Bacillus influenzæ* and the so called Bacillus X, described by Pritchett and Stillman, a few strains of *Bacillus pertussis*, the bacillus of rabbit septicemia, and *Bacillus bronchisepticus* have been included for comparative study.

Upon isolation the majority of the strains of *Bacillus influenzæ* and all the strains of Bacillus X were plated on dextrose agar to which no hemoglobin had been added. In no instance was growth obtained in the hemoglobin-free medium. After prolonged artificial cultivation, in some instances over 2 years, all these strains were again plated on ascitic dextrose agar without hemoglobin. They invariably failed to grow on media which lacked hemoglobin. All media used in this study were enriched by the addition of 4 per cent defibrinated rabbit blood or 2 per cent blood extract. The latter was substituted for defibrinated rabbit blood in the case of the hemolytic Bacillus X, since the hemolysis produced by this organism might mask certain reactions. In many instances also in the work with the non-hemolytic hemophilic bacilli (*Bacillus influenzæ*) when defibrinated blood might interfere with the determination of a reaction, blood extract was used to enrich the media. The extract was made as advised by Wollstein.[9] Defibrinated rabbit blood was boiled for 2 minutes. The clot was finely broken and centrifuged. The resulting extract was added to the media in such a proportion as to give about 2 per cent enrichment. Since Winchell and Stillman[10] found that the optimum hydrogen ion concentration for growth of *Bacillus influenzæ* is between pH 7.3 and 7.5, all media used in the present study had an initial reaction of about pH 7.4 unless otherwise stated.

[9] Wollstein, M., *J. Exp. Med.*, 1919, xxx, 555.

[10] Winchell, A. I., and Stillman, E. G., *J. Exp. Med.*, 1919, xxx, 497.

Non-Hemolytic Hemophilic Bacilli (Bacillus influenzæ).

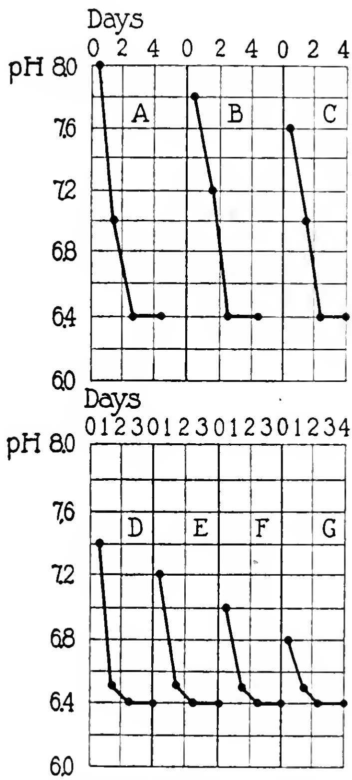
Hydrogen Ion Concentration.—Since the final hydrogen ion concentration reached by growth of an organism in a given medium is a biological constant of differential value, this reaction was determined in the study of the hemophilic bacilli. The colorimetric method, with phenol red and brom cresol purple as indicators, was used to determine the hydrogen ion concentration, and the readings were confirmed by the electrometric method in a number of experiments.

In the usual dextrose meat infusion broth containing 0.5 per cent sodium chloride, *Bacillus influenzæ* attains a final acid reaction of about pH 6.2. If 0.2 per cent sodium phosphate is substituted for the 0.5 per cent sodium chloride in this medium, as is customary for routine purposes in these laboratories, the buffer value is so great that the changes in reaction are insignificant. Since *Bacillus influenzæ* evidently produces relatively small amounts of acid it is desirable in determining the final hydrogen ion concentration to use the medium containing less buffer. Consequently, throughout this work broth containing 0.5 per cent sodium chloride has been used.

In order to determine whether the initial hydrogen ion concentration of the medium had any effect on the final reaction, separate portions of dextrose broth, adjusted to varying hydrogen ion concentrations from pH 8 to 6.8, were inoculated with the same culture of *Bacillus influenzæ*. Text-fig. 1 shows the initial hydrogen ion concentration of the broth when inoculated and the hydrogen ion concentration of the cultures after 20, 44, and 70 hours incubation. From this it is seen that the initial reaction bears no relation to the final hydrogen ion concentration, which is pH 6.4 in each instance.

The relation of oxygen supply to growth was next tested by inoculating a series of 100 cc. Erlenmeyer flasks containing 25 cc. of dextrose broth and a set of large test-tubes containing a similar amount of broth. The initial hydrogen ion concentration of the media was pH 7.3. The test-tube cultures were incubated in an upright position. Colorimetric readings of the hydrogen ion concentrations of the cultures were made after 1, 3, 7, and 14 days incubation. After 24 hours incubation the flask cultures had attained a pH of 6.4, while the test-tube cultures did not reach this end-point until the 7th to 10th day.

It was noted that the macroscopic appearance of the cultures is not a criterion of the hydrogen ion concentration. Cultures which are very turbid and apparently have grown well, when tested may be found not to have reached their lower limit of acid production.



TEXT-FIG. 1. Effect of different initial hydrogen ion concentrations on final hydrogen ion concentrations of *B. influenzæ*.

The final pH was determined on a large series of cultures of *Bacillus influenzæ*. It was found to lie between pH 6 and 6.4. The length of incubation necessary before the different strains, and even the same strain, reach their final hydrogen ion concentration varies. Some strains reach the final reaction at the end of 24 hours; others, at times, do not reach pH 7 even after 14 days incubation in large slanted test-tubes. This variability of growth of *Bacillus influenzæ* has been

encountered throughout the present study. In working with this organism experiments giving negative results must be repeated, since the results may be due merely to insufficient growth.

Sugar Fermentation.—Since all the cultures of *Bacillus influenzæ* ultimately reached a final hydrogen ion concentration of at least pH 6.4 in dextrose broth, a reaction sufficiently acid to be detected by the Andrade indicator, the ability of these organisms to ferment different sugars was tested. It was found that sugar-free broth could not be used as nutrient substrate even after enrichment with blood extract and the addition of the test substance, for *Bacillus influenzæ* did not readily produce acid in this medium. Since meat infusion broth contains varying amounts of muscle sugar which might possibly modify fermentation reactions, Dunham's peptone solution was employed as a basis for the sugars, which were added in 1 per cent concentration. The peptone solution does not contain sufficient reducing sugar to give a positive test with Benedict's reagent. In Dunham's peptone solution enriched with 2 per cent blood extract *Bacillus influenzæ* grows luxuriantly. All culture tubes were incubated in a slanted position so as to expose to the air as large a surface of the medium as possible, since it has been shown that acid production is more rapid under these conditions.

The results of the sugar fermentation are given in Table I. It is seen that almost all strains of *Bacillus influenzæ* produce acid in the monosaccharides, dextrose and galactose. Acid production is less marked and more irregular with levulose. Some strains of *Bacillus influenzæ* fermented the polysaccharides, maltose and saccharose, and to a less extent dextrin. No strains could attack mannitol or lactose. A number of strains were tested against inulin, but the results were so consistently negative that this test substance was subsequently discarded. It is evident that the sugar reactions are irregular. This irregularity is especially noticeable if the same strain is repeatedly inoculated in the same sugar. Although good growth was apparently present, a culture which had been repeatedly positive in dextrose, for instance, at times failed to produce sufficient acid to cause the Andrade indicator to change color. The same factors which sometimes prevented cultures from reaching their final hydrogen ion concentration of pH 6.4 in dextrose broth apparently were acting here.

TABLE I.

Source, Sugar Fermentation, Indole Formation, Nitrate Reduction, and Gas Production of the Strains of Non-Hemolytic Hemophilic Bacilli (B. influenzæ).

Source of strain.	Total No. of strains.	Strains fermenting sugars.																Strains producing indole.		Strains reducing nitrates.		Strains producing gas.		Strains producing hemolysis.	
		Dextrose.		Galactose.		Levulose.		Maltose.		Saccharose.		Dextrin.		Mannitol.		Lactose.									
		No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.
Acute influenza	21	20	95	20	95	13	61.9	4	19	2	9	3	14	0	0	0	0	14	66	21	100	1	4	0	0
Convalescents from influenza	11	11	100	9	82	10	91	0	0	0	0	1	9	0	0	0	0	8	72	11	100	0		0	0
Lobar pneumonia	18	18	100	18	100	8	44	2	11	1	5	1	5	0	0	0	0	12	66	18	100	1	5	0	0
Normal individuals during winter of 1918–19	33	32	97	28	85	24	72	5	15	6	18	4	12	0	0	0	0	18	54	31	94	2	6	0	0
Normal individuals during Sept., 1919	11	10	91	9	82	8	72	5	45	5	45	5	45	0	0	0	0	5	45	11	100	5	45	0	0
Normal individuals during Feb., 1920	25	25	100	24	96	24	96	15	60	16	64	14	56	0	0	0	0	6	24	25	100	7	28	0	0
Total	119	116	97	108	90	87	73	31	26	30	25	28	23	0	0	0	0	63	53	117	98	16	13	0	0

Hydrogen ion determinations were made on a number of strains grown in media containing different sugars. The strains of *Bacillus influenzæ* which showed fermentation with the Andrade indicator had a final hydrogen ion concentration of pH 6 to 6.4 which corresponded to that obtained in dextrose broth when no Andrade indicator was present. An interesting result was the reaction of *Bacillus influenzæ* in lactose. In this sugar there was a definite increase in alkalinity, as illustrated by Table II. If plain peptone solution, peptone solution plus dextrose, and peptone solution plus lactose are inoculated with the same culture of *Bacillus influenzæ* and incubated for 10 days the dextrose culture becomes acid, but the culture in plain peptone and that containing lactose become alkaline. Bacillus X produces a similar increase in alkalinity in lactose media.

TABLE II.

Final Hydrogen Ion Concentration of the Non-Hemolytic Hemophilic Bacilli in Peptone Solution, Peptone Solution Plus Lactose, and Peptone Solution Plus Dextrose.

Solution.	pH
Peptone	8.4+
" + 1 per cent lactose	8.4+
" + 1 " " dextrose	6.0

In the course of the work on sugar fermentation Bronfenbrenner's double indicator "CR" was tested.[11] This indicator is composed of equal parts of a 0.5 per cent aqueous solution of China blue and a 1 per cent alcoholic solution of rosolic acid. China blue appears blue or bluish green in the presence of acid, and colorless in the presence of alkali. Rosolic acid, which is colorless in an acid medium, becomes pink in an alkaline medium.

Dunham's peptone solution containing 1 per cent sugar concentrations and enriched with 2 per cent blood extract was used. 1 per cent CR indicator was substituted for the Andrade indicator previously used. Only two sugars, dextrose and lactose, were tested. Representative strains of the Gram-negative bacilli under observation were used in determining the value of CR as an indicator of acid and alkali production in the presence of these two sugars.

[11] Bronfenbrenner, J., *J. Med. Research*, 1918–19, xxxix, 25.

The hemolytic and non-hemolytic strains of the strictly hemophilic bacilli produced acid in the dextrose CR medium and alkali in the lactose CR medium as indicated by the definite color changes after 48 hours incubation. The intensity of the reaction increases with prolonged incubation. The bacillus of rabbit septicemia also produced acid in the presence of dextrose and alkali in the presence of lactose. The strains of *Bacillus pertussis* and *Bacillus bronchisepticus* produced alkali in both the dextrose and lactose media. These results correspond exactly with the results obtained with similar sugar media in which Andrade indicator replaced CR, as will be seen by referring to Tables I, III, and IV.

CR, in the concentration used, does not appear to be bactericidal for any of the strains tested in the fluid medium described, for good growth was obtained in each instance and the color changes which occurred were striking. In poured oleate hemoglobin agar plates to which CR and the desired sugar had been added in 1 per cent concentration, striking color changes did not occur. In the concentration used in solid medium CR did not appear to be of value as a differential indicator.

Indole Production.—Jordan has called attention to the production of indole by *Bacillus influenzæ*. The indole production by cultures included in this study was tested by Ehrlich's para-dimethylaminobenzaldehyde method. Of the 119 strains of *Bacillus influenzæ* studied, 63, or 53 per cent, produced indole. It was found that indole was present at times after only 18 hours incubation at 37°C. and was produced for as long a period as 3 weeks. Indole was produced quite regularly in plain blood broth cultures, but slightly more positive reactions were obtained if Dunham's peptone solution enriched with blood extract was used. This may be due to the fact that the defibrinated blood masked delicate reactions. The same irregularity of reaction that was noted in the fermentation of sugar by *Bacillus influenzæ* was observed in the production of indole; occasionally an indole-producing strain, which apparently had grown luxuriantly, failed to produce indole. Hence the necessity of repeated tests before a culture may be definitely classified as a non-indole producer. A possible relation exists between indole production and sugar fermentation. Only one indole-producing strain fermented the polysaccharides.

Nitrate Reduction.—Of the 119 strains of *Bacillus influenzæ* studied, 117 were able to reduce nitrates to nitrites. This reduction as a rule occurred after 24 hours incubation. Like other reactions with these delicate organisms, at times negative results were obtained without apparent reason. In a few instances a culture which had given a positive nitrite reaction after 24 hours incubation failed to give it after 10 days incubation.

Gas Production.—The ability of the hemophilic bacilli to produce gas was first tested by making stab cultures in 1 per cent dextrose agar to which a small amount of blood extract had been added. Under these partial anaerobic conditions luxurious growth was obtained with all strains. Of the 119 non-hemolytic strains, sixteen, or 13 per cent, were found to produce gas. The gas appeared usually after 48 to 72 hours incubation, but with several strains did not appear until later. The time of incubation necessary before the appearance of gas varied on different occasions when the same strain was used. The amount of gas produced was never very great.

Stab cultures were made with blood extract dextrose medium containing agar in concentrations of 1, 1.5, and 2 per cent. 1 per cent agar seemed to be the most favorable concentration for the demonstration of gas. Gas production was demonstrated also in shake cultures of dextrose blood extract agar. With the exception of one strain, the non-hemolytic gas-producing organisms did not produce gas in Smith fermentation tubes when meat infusion broth or Dunham's peptone solution containing dextrose and blood extract was used. It appears, therefore, that a solid medium is more suitable than a fluid medium for the production of gas by these non-hemolytic strains of hemophilic bacilli.

Hemolytic Hemophilic Bacilli (*Bacillus X*).

The hemolytic hemophilic bacilli reach a final hydrogen ion concentration which varies from pH 6.4 to 5.8. Most strains ferment dextrose, maltose, and saccharose readily and quite regularly, and utilize galactose, levulose, and dextrin less easily and more irregularly. The same irregularities and difficulties of growth have been encountered with this organism as with the non-hemolytic type, although it is not quite so variable. Of the twenty-nine hemolytic strains studied only

three produced indole after repeated attempts. All the strains reduced nitrates to nitrites. Only four strains, or 13 per cent, showed the production of gas in dextrose blood extract agar. With these organisms the gas appeared usually after 24 to 48 hours of incubation. With only one of the strains could gas production be demonstrated in a Smith tube with meat infusion broth containing dextrose and blood extract. Stab and shake cultures were made in a similar manner as described above in connection with the non-hemolytic organisms.

TABLE III.

Sugar Fermentation, Indole Formation, Nitrate Reduction, and Gas Production of the Strains of Hemolytic Hemophilic Bacilli (Bacillus X) Isolated from Normal Individuals during the Winter of 1919–20.

Total No. of strains.	Strains fermenting sugars.																		Strains producing indole.		Strains reducing nitrates.		Strains producing gas.		Strains producing hemolysis.	
	Dextrose.		Galactose.		Levulose.		Maltose.		Saccharose.		Dextrin.		Mannitol.		Lactose.		Inulin.									
	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.
29	29	100	3	10	9	31	24	82	23	79	15	51	0	0	0	0	0	0	3	10	29	100	4	13	29	100

Relation between Indole Formation and Gas Production.

An apparent relation between indole formation and gas production can be observed. All the non-hemolytic gas-producing strains are non-indole producers and comprise strains that ferment mono- as well as polysaccharides. The hemolytic gas-producing strains, with one exception, produce indole. These hemolytic gas-producing strains do not ferment sugars so readily as the other organisms in this group.

Comparative Study of Bacillus pertussis, the Bacillus of Rabbit Septicemia, and Bacillus bronchisepticus.

Table IV shows the results of a comparative study of *Bacillus pertussis*, the bacillus of rabbit septicemia, and *Bacillus bronchisepticus* in connection with the hemophilic bacilli.

TABLE IV.

Sugar Fermentation, Indole Formation, Nitrate Reduction, and Gas Production of Strains of B. pertussis, the Rabbit Septicemia Bacillus, and B. bronchisepticus.

Bacillus.	Total No. of strains.	Strains fermenting sugars.																		Strains producing indole.		Strains reducing nitrates.		Strains producing gas.		Strains producing hemolysis.	
		Dextrose.		Galactose.		Levulose.		Maltose.		Saccharose.		Dextrin.		Mannitol.		Lactose.		Inulin.									
		No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.
B. pertussis	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rabbit septicemia bacillus	4	4	100	4	100	4	100	0	0	4	100	0	0	4	100	0	0	0	0	4	100	2	50	0	0	0	0
B. bronchisepticus	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Bacillus pertussis.—The four strains of *Bacillus pertussis* studied were stock cultures which had been under artificial cultivation for a considerable length of time. They had a final hydrogen ion concentration of pH 8 to 8.6 in dextrose broth and failed to produce acid in any of the sugars tested. Neither did they produce indole or reduce nitrates to nitrites. In dextrose agar stab cultures there was only a slight growth below the surface and no gas was produced.

Bacillus of Rabbit Septicemia.—The four strains of the bacillus of rabbit septicemia had a final hydrogen ion concentration of pH 6 and produced acid in dextrose, galactose, levulose, saccharose, and mannitol. They did not produce acid in maltose, lactose, dextrin, or inulin. These organisms produced indole slowly, and two strains, or 50 per cent, reduced nitrates to nitrites. In dextrose agar stab cultures there was good growth in the stab, but no gas was produced.

Bacillus bronchisepticus.—The two strains of *Bacillus bronchisepticus* studied had a final hydrogen ion concentration of pH 9.2 in dextrose broth and failed to produce acid in any of the sugars tested. They did not produce indole or reduce nitrates to nitrites. In dextrose agar stab cultures there was only a slight growth below the surface and no gas was produced.

DISCUSSION.

The small Gram-negative hemophilic bacilli which have gradually come to be considered as belonging to one group of organisms and to which the name *Bacillus influenzæ* has been given, appear in the light of the present study to be rather a group of closely allied bacilli which have demonstrable biological differences. The bacillus which Pfeiffer first described and associated with clinical influenza is now questioned as being the etiological factor in the spread of this disease. However, the percentage of cases in which the bacillus of Pfeiffer has been recovered is great enough to indicate that this organism may be at least a secondary invader. Since the first description of this hemophilic bacillus in 1892 by Pfeiffer, little has been added to our knowledge of its biological characteristics.

In this study we have found that the hemophilic bacilli observed divide themselves naturally into two large groups according to their

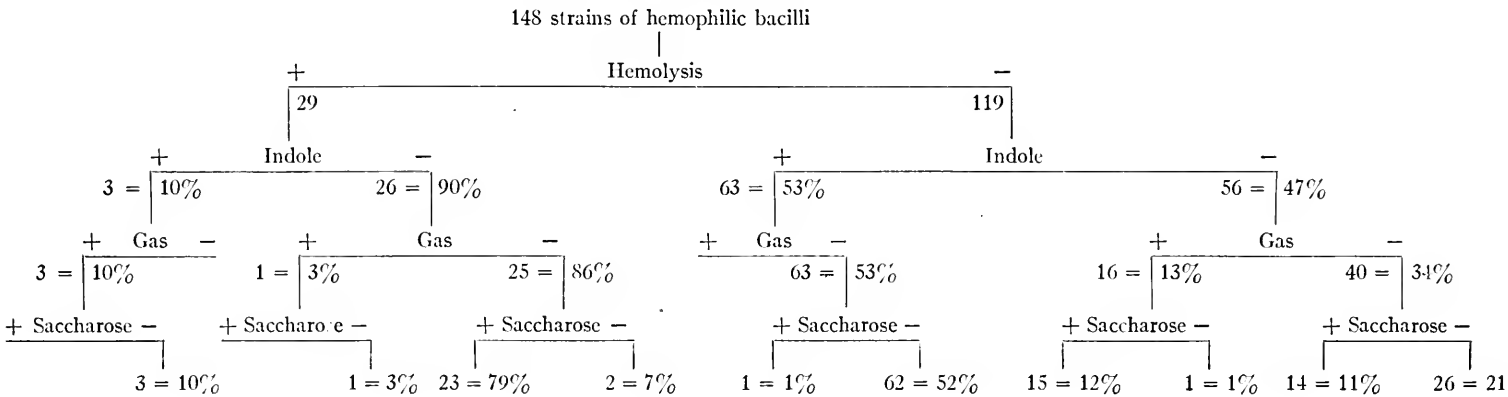
ability to hemolyze whole blood. The hemolytic group comprises the organisms originally described as Bacillus X by Pritchett and Stillman, and occurs in normal mouths. Many of these hemolytic bacilli have no doubt been confused with the non-hemolytic variety due to the almost universal use of chocolate medium. On oleate agar the colonies are so similar that they cannot be distinguished, and morphological differences are so slight as not to be reliable. Organisms of the hemolytic type (Bacillus X) do not live so long in culture media as those of the non-hemolytic type. They are best preserved at a low temperature. A few strains have been found to live from 2 to 3 weeks if kept in blood broth in the ice chest, but in order to be successfully preserved in stock cultures they must be transplanted every 6 or 7 days. At room temperature Bacillus X survives about 5 days, while at 37.5°C. it remains viable about 10 days. The non-hemolytic group (*Bacillus influenzæ*), on the other hand, remains viable for a month or more at room temperature in blood broth.

The hemolysin produced by the hemolytic type is quite stable, retaining its activity after being kept on ice for 6 weeks to several months. It can be demonstrated in a young broth culture after 2 hours incubation at 37°C. It is non-filterable and is destroyed by heating for $\frac{1}{2}$ hour at 56°C. Different strains vary, however, in their ability to produce hemolysis. The hemolytic bacilli are non-pathogenic for rabbits, guinea pigs, and mice.

Both the non-hemolytic and hemolytic groups of hemophilic bacilli attain a final hydrogen ion concentration of approximately pH 6.4, although the hemolytic group may reach pH 5.8. Both produce acid in dextrose, but in both groups only certain strains ferment saccharose. The greater ability of the hemolytic organisms to ferment sugars may be a basis for further differentiation.

A tentative classification, graphically illustrated below, defines a small subgroup of the hemolytic group formed by the strains which produce indole and gas but do not ferment saccharose. These strains appear to ferment sugars less readily and require further study to determine whether the indole-producing strains are also gas producers. The greater number of hemolytic strains, however, do not produce indole or gas, but ferment saccharose.

Tentative Classification of the Hemophilic Bacilli Based on Hemolysis, Indole and Gas Production, and the Fermentation of Saccharose.



The non-hemolytic organisms are subdivided into two fairly even groups comprising indole-producing and non-indole-producing strains. None of the indole producers forms gas, in contrast with the hemolytic group. With one exception, none of the non-hemolytic indole-producing strains ferments saccharose. A large majority of the non-indole-producing organisms of the non-hemolytic type do not form gas and do not ferment saccharose. With a single exception, all the indole-negative strains which form gas also ferment saccharose.

One of the most striking features of this classification may be best illustrated by Table V, which represents a comparison of three factors differentiating the hemolytic and non-hemolytic groups of the hemophilic bacilli. Here it is seen that the majority of the strains of the hemolytic type do not produce indole or gas, but ferment saccharose,

TABLE V.

Three Differential Factors of the Hemolytic and Non-Hemolytic Groups of the Hemophilic Bacilli.

Differential factors.	Hemolytic group.		Non-hemolytic group.	
	Positive.	Negative.	Positive.	Negative.
	per cent	*per cent*	*per cent*	*per cent*
Saccharose	79	21	25	75
Gas	13	86	13	87
Indole	10	90	53	47

while the reverse is true of the non-hemolytic type; that is, the majority of the non-hemolytic organisms do not produce gas but also do not ferment saccharose. It will be noted that the non-indole and indole-producing strains of the non-hemolytic type fall into almost even groups.

The classification made in this study is merely a tentative one. Undoubtedly when the technique of the reactions is more nearly perfected and a larger number of hemophilic bacilli has been studied, the group differentiations will be more striking and regular.

Although the number of strains of *Bacillus influenzæ* employed is too small to warrant any definite conclusions, it would seem that the non-hemolytic bacilli isolated from individuals suffering with and recovering from respiratory infections and those isolated from normal mouths during the epidemic period differ biologically in certain re-

spects from the strains recovered from normal individuals during the winter of 1919–20. This point is illustrated by Table VI. It is seen that the group of non-hemolytic hemophilic bacilli recovered from normal mouths during the winter of 1919–20 shows a higher percentage of strains which ferment the polysaccharides, maltose, saccharose, and dextrin, and more strains which produce gas, but fewer indole-producing strains.

TABLE VI.

Comparison of the Strains of Non-Hemolytic Hemophilic Bacilli Recovered from Respiratory Infections and Normal Mouths during Epidemic Period of 1918 and Strains Recovered from Normal Individuals during the Winter of 1919–20.

Source of strain.	Total No. of strains.	Strains fermenting sugars.												Strains producing indole.		Strains producing gas.	
		Dextrose.		Galactose.		Levulose.		Maltose.		Saccharose.		Dextrin.					
		No.	Per cent.	No.	Per cent.	No.	Per cent.	No	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.
Respiratory diseases and normal mouths during epidemic of 1918, and respiratory diseases during 1919–20.	83	81	97	75	90	55	66	11	13	9	10	9	10	52	62	4	4
Normal mouths during winter of 1919–20.	36	35	97	33	91	32	88	20	55	21	58	19	52	11	30	12	33

The Gram-negative bacilli which are not hemophilic and which have been studied because of their morphological similarity can be easily differentiated from the hemolytic and non-hemolytic hemophilic bacilli of the influenza type. The bacillus of rabbit septicemia shows a striking similarity to members of the non-hemolytic hemophilic group in the limiting hydrogen ion concentration, indole production, and nitrate reduction. On the contrary, *Bacillus pertussis* and *Bacillus bronchisepticus*, while resembling each other in certain reactions, do not simulate the strictly hemophilic group. These organisms have a markedly alkaline final hydrogen ion concentration, and do not produce indole or reduce nitrates.

CONCLUSIONS.

1. The hemophilic bacilli can be divided into two large groups according to the ability of certain strains to produce hemolysis.

2. Both the hemolytic and the non-hemolytic groups may be further subdivided according to the ability of some strains to produce indole, to form gas, and to ferment certain carbohydrates.

3. The hemophilic bacilli of both the hemolytic and the non-hemolytic varieties when grown in meat infusion broth containing 1 per cent of dextrose reach a final hydrogen ion concentration of about pH 6.4. In addition, practically all the strains possess the power to reduce nitrates to nitrites.

We wish to express our thanks to Dr. N. S. Ferry for a culture of *Bacillus bronchisepticus*, to Dr. Martha Wollstein for cultures of *Bacillus pertussis* and the bacillus of rabbit septicemia, to Dr. Anna Williams for cultures of *Bacillus pertussis*, to Dr. F. S. Jones for a culture of *Bacillus bronchisepticus*, and to the American Museum of Natural History for a culture of *Bacillus pertussis*.

FURTHER STUDIES ON THE ETIOLOGICAL RÔLE OF VIBRIO FETUS.*

By THEOBALD SMITH, M.D., RALPH B. LITTLE, V.M.D., and MARIAN S. TAYLOR.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

(Received for publication, July 8, 1920.)

Among the twenty-six cases of abortion associated with *Vibrio fetus*,[1, 2] of which one also contained *Bacillus abortus*, there was none involving heifers. All were second or later pregnancies. All but three were purchased cows. More recently three cases of abortion in native heifers have been found associated with *Vibrio fetus*. These cases are of sufficient importance to be given in detail.

No. 433.—Native heifer; aborted October 24, 1919. Records state that she had been bred February 22, 1919. Male fetus, 28 inches long; color black and white. Coat of hair still lacking. The stomach fluids contained a few particles of meconium only. The contents of the large intestine were normal. The lungs were free from air. There were some small hemorrhages in the auriculo-ventricular valves. Besides a general fullness of blood in the organs there were no marked abnormalities in the fetus.

Cultures were made as follows: From the contents of the fourth stomach, which showed spirals in films, pure cultures of spirilla were obtained. Films from small and large intestines were negative, but cultures developed spirilla from contents of the colon and rectum. Cultures from the lungs and spleen also contained spirilla only. Those from the liver and kidneys remained sterile.

One guinea pig was inoculated with the contents of the fourth stomach, another with meconium, and a third with lung tissue. Killed after 7 weeks, none showed any signs of disease due to *B. abortus*, and the spleen cultures remained sterile.

A portion of the placenta representing the unoccupied horn, which had passed out, was cut from the still adherent and retained remainder. It was covered with shavings and without any odors of decomposition. During the washing to

* The tests relating to the milk were made by Miss Marion L. Orcutt.

[1] Smith, T., *J. Exp. Med.*, 1918, xxviii, 701.

[2] Smith, T., *J. Exp. Med.*, 1919, xxx, 313.

remove adherent bedding, yellowish, cheesy particles were carried off by the wash water from the cotyledons.

The chorion varies from a smooth, translucent, slightly injected membrane to one opaque, thickened, and leathery. The opacity is due to an infiltration which is in the form of slightly elevated plaques not removable by gentle scraping. In other places the infiltration is discrete, in the form of whitish opacities, $\frac{1}{2}$ to 1 mm. in diameter. There are also scattering minute tufts of adventitious villi, completely cheesy. Edema of the subjacent tissue varies in thickness from place to place. The cotyledons are in part normal, in part diseased. Some of the latter are yellowish, pultaceous throughout. The still normal cotyledons contain, usually on the margin, necrotic, yellowish villi. More rarely such villi are scattered through the cotyledon.

Many films were examined without showing any bacilli of the type of *B. abortus.* There were, however, occasional larger rods and vibrios. A few were closely wound, the rest flatter, as is usual with *V. fetus.*

Sections of fixed and hardened material from various regions of the placenta show necrosis of villi, loss of surface epithelium with infiltration of the underlying tissue with leucocytes in certain areas. Where epithelium is present, no bacteria are found in them, as is the case when *B. abortus* is present. The endothelium of the capillaries has proliferated in places and it partly or nearly fills the lumina. Bacteria resembling vibrios are detected within these cells and in groups among necrotic villi. Inoculation of two guinea pigs with scrapings from the diseased placenta was negative as regards *B. abortus.*

Milk collected 3 days after the expulsion of the fetus, centrifuged according to the method previously described,[3] and injected into three guinea pigs also yielded negative results as regards *B. abortus.* Agglutinins for *B. abortus* were absent from the milk at this time.

The blood agglutinations of this heifer are significant in that they indicate subsequent infection with *B. abortus* during the second pregnancy. 2 days before abortion, October 22, 1919, the titer limit was 1: 20, 20 days after abortion a trifle above 1: 40. Nearly 6 months later samples taken 4 days apart were 1: 640.

No. 438.—Date of breeding uncertain. Aborted November 9, 1919. The placenta was retained. Fetus, female, measures $17\frac{1}{2}$ inches and weighs about 5 pounds. No subcutaneous edema or serous effusions. Autolytic changes of organs without any distinctly putrefactive odors present. The fourth stomach contains a thick mucoid pinkish fluid and films show spirilla. Films of contents of colon and rectum do not show any bacteria. One ventral lobe of the lungs shows under pleura fine, branching, grayish yellow lines made up of masses of spherical crystals.

Cultures from contents of the fourth stomach contain only spirilla. The same is true of cultures of the colon contents. One culture from the rectum contains

[3] Smillie, E. W., Little, R. B., and Florence, L., *J. Exp. Med.*, 1919, xxx, 341.

cocci, the other only spirilla. Cultures made with bits of spleen, liver, and lungs develop only spirilla. One tube from the kidney contains large bacilli, the other only spirilla.

Four guinea pigs, inoculated respectively with a suspension of material from a uterine swab, with fourth stomach contents, meconium, and lung tissue, were negative as to *B. abortus* when killed and cultured after 6 to 7 weeks.

A sample of milk drawn 9 days after expulsion of the fetus was inoculated into three guinea pigs. *B. abortus* was not isolated from any of them. The titer limit of the agglutinations for *B. abortus* of this heifer 4 days after abortion was 1:40, the milk titer was negative when tested 9 days after. 5 months after abortion the titer limit of the blood was 1:80, of the milk below 1:20.

No. 449.—Aborted during the night of December 1 to 2, 1919. Date of breeding unknown. Female fetus, 16 inches long. Lower jaw and part of upper jaw eaten away by some animal during the night. Some coils of the small intestine protruding from the opening at the umbilicus. General suffusion of tissues with hemoglobin. Autolytic changes indicated by appearance of tissues. Skin readily peeled away from subjacent muscular tissue.

Contents of the fourth stomach turbid. They contain epithelial growths from amnion, swallowed by the fetus. Films from the fourth stomach contents contain numerous spirilla. One culture from the contents contains only spirilla. In the other they are associated with bacilli. Cultures from the liver contain only spirilla. Those from the lungs contain a mixture of rods and cocci.

Only one guinea pig was inoculated with the contents of the fourth stomach. The result was negative as to *B. abortus*.

Milk collected 1 day after expulsion of the fetus was tested on three guinea pigs for *B. abortus* with negative result.

The agglutination titer of this heifer also indicated absence of *B. abortus*. On February 12, 1919, and April 1, it was 1:80. On November 25 it was 1:20. On the date of abortion, December 2, it was 1:40. $4\frac{1}{2}$ months later it was 1:80. The agglutination titer limit of the milk 1 day after abortion was 1:40, $4\frac{1}{2}$ months later it was below 1:20.

Agglutination Tests.

It was stated in an earlier publication[4] that the agglutinability of recently isolated strains of *Vibrio fetus* is low or absent and that it rises under artificial cultivation to a certain maximum. This is not always true, however, as Table I shows. The vibrios of the three aborting cases are titrated against the serum of a rabbit treated repeatedly with cultures of Strain 67. In all cases a high agglutina-

[4] Smith, T., and Taylor, M. S., *J. Exp. Med.*, 1919, xxx, 299.

tion titer is demonstrated, although the strains are still in the early generations. The table also shows a general serological relationship among the strains tested.

The outcome of several tests of the blood serum of the three reported cases is summarized in Table II. Owing to a slight spontaneous agglutination in the controls, the high titers with Vibrio 356 will have to be reduced one or two degrees. The results, while not uniform, give encouragement that agglutination tests may be of use should this type of infectious abortion become more widespread. In only one case (No. 449) was serum antedating the abortion on hand, and this was negative. We have, therefore, no way of knowing at present whether a high titer precedes abortion, as is almost regularly true in the presence of *Bacillus abortus*. By choosing some easily agglutinable strain which has been tested against normal sera it may be possible to detect this type of infection as readily as the other. All of the strains included in the table were tested against the serum of a normal heifer and no clumping was observed in dilutions ranging from 1:20 to 1:1,280.

TABLE I.

Agglutination Tests with Serum from a Rabbit Immunized with Cultures of Vibrio 67.

Vibrio No.	No. of transfer.	Serum dilutions.								Control.
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	Vibrios in salt solution.
433	11th	C.*	C.	C.	C.	C.	C.	+++	+++	–
438	9th	"	"	"	"	"	"	C.	+++[1]	–
449	5th	"	"	"	"	"	"	"	+++	–
67	125th	"	"	"	"	"	"	"	+++	–

* C. indicates complete agglutination, +++ nearly complete agglutination, ++ marked agglutination, + slight agglutination, ± doubtful, and – no agglutination.

[1] when following a symbol denotes a degree of agglutination between it and the next higher symbol.

TABLE II.

Agglutination Tests with Blood Serum of the Three Cases.

Case No.	Date of abortion.	Date of withdrawal of blood.	Vibrio used as antigen.		Date of test.	Serum dilutions.								Controls.	
			Case No.	Transfer.		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	Vibrios in salt solution.	Normal serum.
433	Oct. 24, 1919	Nov. 13, 1919	433	6th	Dec. 1, 1919	+++[1]	+++[1]	+++[1]	+++[1]	+++	++	–	–	–	–
		" 13, 1919	347	27th	" 1, 1919	+++[1]	+++[1]	+++	++	–	–	–	–	–	–
		Apr. 15, 1920	433	16th	Apr. 19, 1920	C.	C.	+++[1]	+++	+++	++	+	±	±	–
		" 15, 1920	356	32nd	" 19, 1920	+++[1]	+++[1]	+++[1]	+++[1]	+++[1]	+++	+++	+	+	–
438	Nov. 9, 1919	Nov. 13, 1919	438	4th	Dec. 1, 1919	++	+++[1]	+++[1]	+++[1]	+++	++	–	–	–	–
		" 13, 1919	347	27th	" 1, 1919	+++	+++	+++	++	–	–	–	–	–	–
		Apr. 15, 1920	449	16th	Apr. 19, 1920	C.	C.	+++[1]	+++	–	–	–	–	–	–
		" 15, 1920	356	32nd	" 19, 1920	"	+++[1]	+++[1]	+++[1]	+++[1]	+++	+++	++	+	–
449	Dec. 1, 1919	Nov. 25, 1919	449	7th	Jan. 31, 1920	–	–	–	–	–	–	–	–	–	–
		" 25, 1919	347	33rd	" 31, 1920	++	+	–	–	–	–	–	–	–	–
		Dec. 2, 1919	449	5th	" 19, 1920	++[1]	–	–	–	–	–	–	–	–	–
		" 2, 1919	347	33rd	" 31, 1920	+++	++	+	–	–	–	–	–	–	–
		Apr. 15, 1920	449	13th	Apr. 19, 1920	++	–	–	–	–	–	–	–	–	–
		" 15, 1920	356	32nd	" 19, 1920	+++[1]	+++	+++	+++	+++	++[1]	++	+	+	–

GENERAL SUMMARY.

The data bearing on these three cases are quite sufficient to rule out *Bacillus abortus* as the agent. Not only the cultures and guinea pig tests of fetal tissues and contents of the digestive tract, but also the agglutination and guinea pig tests of the milk, were negative. The same is true of the agglutination tests of the blood serum. Only in one case was the placenta obtained in part. The stained films and the sections from various regions showed no abortion bacilli. Guinea pig tests of placental tissue were negative for *Bacillus abortus*. On the other hand, minute organisms resembling vibrios were detected in the cytoplasm of endothelial cells within capillaries in the edematous subchorionic tissue. Subsequently the agglutination titer of the blood serum of one of these cases rose to a level indicating infection with *Bacillus abortus* during the second pregnancy.

The peculiar distribution of abortions due to *Vibrio fetus* among older cows and heifers in this herd, resulting at first in cases among older cows and latterly passing to young stock, may be explained by certain occurrences in the herd itself. It may be assumed that the infection was originally brought in by purchased cows. The young stock is kept segregated from these in a special barn, and when 6 months old it is pastured on outlying farms until returned in an advanced stage of pregnancy. The heifers during the first pregnancy were thus kept away from vibrio carriers until after the first calf was born.

In June and July, 1919, 55 older cows, purchased and native, were placed on the young stock pasture. The three cases of abortion in heifers due to *Vibrio fetus* occurred October 24, November 9, and December 2, 1919. The age and condition of the fetuses accord very well with the assumption that *Vibrio fetus* was introduced among the young stock in June or July of the same year.

The information gathered thus far concerning vibrionic abortion in this herd enables us to formulate a tentative hypothesis subject to modification with increasing knowledge of this type of infectious abortion. The infectious agent was probably introduced by purchased cows in 1917 or earlier. It gained a certain headway up to 1919, then the number of cases declined so that between May, 1919,

and May, 1920, only the above three cases in heifers, and one case of mixed infection with *Bacillus abortus* in an older cow, were detected. During the same period cases due to *Bacillus abortus* continued undiminished. The greater resistance of *Bacillus abortus* manifested in cultures as compared with *Vibrio fetus* is thus reflected in its behavior in nature. The temporary dying out of the infection indicates that natural immunization of a herd to *Vibrio fetus* proceeds quite rapidly. Another outbreak may be expected when the immunity of the herd has declined in the absence of the infecting agent and the latter is reintroduced from without, or it may reappear at any time when a vibrio of higher virulence is brought in.

STUDIES ON EXPERIMENTAL PNEUMONIA.

IX. Production in Monkeys of an Acute Respiratory Disease Resembling Influenza by Inoculation with Bacillus influenzæ.

By FRANCIS G. BLAKE, M.D., and RUSSELL L. CECIL, M.D.

(*From the Bacteriological Laboratories of the Army Medical School, Washington.*)

(Received for publication, May 15, 1920.)

The present paper deals with the production in normal monkeys of an acute respiratory disease by inoculation of the mucous membranes of the upper respiratory tract with *Bacillus influenzæ* and of bronchopneumonia by intratracheal injection.[1] These experiments were undertaken in continuation of previous studies[2, 3, 4] on experimental pneumococcus and hemolytic streptococcus pneumonia in monkeys with the purpose of obtaining evidence concerning the relation of *Bacillus influenzæ* to influenza and of determining whether the type of bronchopneumonia that has been ascribed[5, 6, 7] to *Bacillus influenzæ* infection of the lungs in man could be reproduced experimentally in animals.

In general, animal inoculation experiments with *B. influenzæ*, though serving to demonstrate[8,9,10] the pathogenicity of at least some strains of the organism for laboratory animals, have had little actual bearing on the relation of *B. influenzæ* to influenza, since the methods of inoculation have been by the subcutaneous, intraperitoneal, or intravenous routes, and it is not to be expected that a disease

[1] Blake, F. G., and Cecil, R. L., *J. Am. Med. Assn.*, 1920, lxxiv, 170.

[2] Blake, F. G., and Cecil, R. L., *J. Exp. Med.*, 1920, xxxi, 403.

[3] Blake, F. G., and Cecil, R. L., *J. Exp. Med.*, 1920, xxxi, 445.

[4] Blake, F. G., and Cecil, R. L., *J. Exp. Med.*, 1920, xxxii, 401.

[5] Pfeiffer, R., *Z. Hyg. u. Infectionskrankh.*, 1893, xiii, 357.

[6] MacCallum, W. G., *J. Am. Med. Assn.*, 1919, lxxii, 720.

[7] Wolbach, S. B., *Bull. Johns Hopkins Hosp.*, 1919, xxx, 104.

[8] Cohen, *Ann. Inst. Pasteur*, 1909, xxiii, 273.

[9] Wollstein, M., *Am. J. Dis. Child.*, 1911, i, 42; *J. Exp. Med.*, 1915, xxii, 445.

[10] Albert, H., and Kelman, S. R., *J. Infect. Dis.*, 1919, xxv, 443.

which may safely be classified as a respiratory infection could readily be reproduced in animals by introduction of the virus by other than the natural path of infection in man. Attempts have been made, however, to produce influenza by inoculation of the mucous membranes of the respiratory tract with *B. influenzæ*. Pfeiffer[5] inoculated monkeys and other laboratory animals on the mucous membranes of the upper respiratory tract, intratracheally, and by direct injection into the lung, but failed satisfactorily to reproduce influenza or to establish the fact that actual infection took place, though many of the animals suffered from a temporary intoxication and sometimes died. Wollstein and Meltzer[11] produced bronchopneumonic lesions in dogs by massive intrabronchial insufflation of *B. influenzæ*. Wahl, White, and Lyall[12] inoculated the nasal and nasopharyngeal mucous membranes of healthy men with four strains of *B. influenzæ* with negative results. Similar experiments conducted by the United States Public Health Service[13] likewise yielded negative results.

Strain of Bacillus influenzæ Used.

Since it seemed apparent that there was no adequate method available for distinguishing between possible saprophytic and pathogenic varieties of Gram-negative hemophilic bacilli to which the name *Bacillus influenzæ* is at present indiscriminately applied, it seemed desirable to employ a single strain which could be considered with reasonable certainty to be the infecting organism in the case from which it was isolated. Accordingly, the strain of *Bacillus influenzæ*[14] used, designated B, was one originally isolated in pure culture from the pleural exudate withdrawn from a case of empyema following influenzal pneumonia in a child. It conformed to such criteria as we possess for identifying *Bacillus influenzæ* in that it was a small, Gram-negative, non-motile, non-spore-bearing, non-hemolytic bacillus, growing readily in characteristic colonies on suitable hemoglobin-containing media at 37°C., but failing to grow in the absence of hemoglobin or at 20°C. It produced indole. When received it had been grown on artificial media for about 6 weeks and was found to possess practically no virulence for white mice, the 18 hour growth on a whole brown blood agar plate not being lethal on intraperitoneal inoculation.

[11] Wollstein, M., and Meltzer, S. J., *J. Exp. Med.*, 1912, xvi, 126.

[12] Wahl, H. R., White, G. B., and Lyall, H. W., *J. Infect. Dis.*, 1919, xxv, 419.

[13] *Public Health Rep., U. S. P. H.*, 1919, xxxiv, 33.

[14] We are indebted to Dr. T. M. Rivers of the Johns Hopkins Medical School, Baltimore, for the strain of *B. influenzæ* used.

Method of Increasing the Virulence of Bacillus influenzæ.

A preliminary intratracheal test inoculation of a monkey having resulted in no evidence of infection, it was evident that the virulence of the strain of *Bacillus influenzæ* would have to be raised if successful results were to be obtained. This was accomplished by successive animal passages through a series of eleven white mice followed by a series of thirteen monkeys. These animals were inoculated intraperitoneally with broth or 0.85 per cent salt solution suspensions of *Bacillus influenzæ* made from brown blood agar plate cultures. At the beginning of the mouse series the growth from one-half of a plate culture was inoculated. 6 to 8 hours after inoculation the mouse was killed and a plate culture of the peritoneal exudate was made for inoculation of the next mouse on the following day. This procedure was adopted because a few preliminary experiments had shown that the influenza bacillus began to disappear from the peritoneal exudate by 8 hours after inoculation and was entirely gone by 24 hours. With the successive inoculations the organism became progressively more virulent for mice until at the end of the series the growth on one-fourth of a plate killed a mouse in 6 to 8 hours with peritonitis and septicemia.

Following the mouse series the organism was passed by similar methods through thirteen monkeys. The monkeys received large inoculations varying from the growth on four to that on fourteen plates. Cultures for the next succeeding inoculation were made either from peritoneal exudate withdrawn by a sterile capillary pipette 6 to 8 hours after intraperitoneal injection, or from the peritoneal exudate at autopsy in the monkeys that succumbed. Some delay occurred in this series owing to secondary invasion of the peritoneal cavity by *Staphylococcus albus* in some of the monkeys. When this occurred it was necessary to prepare subcultures for the next passage in order to eliminate the staphylococcus. The symptoms produced by the inoculations were those of peritonitis and profound intoxication. In monkeys in which leucocyte counts were made a rapidly developing and very marked leucopenia was found, the counts falling in 3 to 6 hours after inoculation from the normal range of 15,000 to 25,000 down to 2,500 to 10,000 per c. mm., and remaining low until death or recovery took place. The series of passages is shown in Table I.

TABLE I.

Passage of B. influenzæ through Monkeys by Intraperitoneal Inoculation.

Passage monkey No.	Date of inoculation.	Culture of *B. influenzæ*.	Amount inoculated.	Result.	Culture of peritoneal exudate. 6–8 hrs. after inoculation.	Culture of peritoneal exudate. Autopsy.
	1919					
P 1	July 29	B. 11[1]*	7 plates.	Recovered.	*B. influenzæ.*	
" 2	" 30	" 11.1[1]	4 "	"	" "	
" 3	Aug. 2	" 11.2[3]	9 "	Aug. 3. Died.	*Staphylococcus albus.* *B. influenzæ.*	*B. influenzæ.*
" 4	" 3	" 11.3[1]	$5\frac{1}{2}$ "	" 4. "	" "	" "
" 5	" 6	" 11.4[2]	8 "	" 7. "	" "	*Staphylococcus albus.* *B. influenzæ.*
" 6	" 7	" 11.5[1]	5 "	Recovered.	" "	
" 7	" 9	" 11.6[2]	12 "	Aug. 10. Died.	" "	*B. influenzæ.*
" 8	" 12	" 11.7[2]	11 "	" 14. "	" "	" "
" 9	" 14	" 11.8[2]	12 "	" 15. Killed.	" "	" "
" 10	" 16	" 11.9[2]	14 "	Recovered.	" "	
" 11	" 19	" 11.10[2]	10 "	"	*Staphylococcus albus.* *B. influenzæ.*	
" 12	" 20	" 11.11[1]	10 "	Aug. 20. Killed.		*B. influenzæ.*
" 13a	" 22	" 11.12[2]	10 "	Recovered.	*B. influenzæ.* Diphtheroid bacillus.	
" 13b	" 25	" 11.12[2]	12 "	Aug. 26. Died.		*B. influenzæ.*

* B. 11 indicates *B. influenzæ*, Strain B, 11 mouse passages. B. 11.1, etc., indicate Strain B, 11 mouse passages, 1 monkey passage, etc. The exponents indicate the number of cultures removed from the last passage animal.

At the completion of the series 0.1 cc. of a 16 hour blood broth culture of *Bacillus influenzæ* from the peritoneal exudate of Passage Monkey 13b killed a white mouse within 48 hours, 1 cc. in 5 hours. To indicate the rapidity with which this increased virulence was lost by subculture on artificial media, it may be stated that after five transplants in blood broth at 24 hour intervals 1 cc. failed to kill a mouse.

Method.

Two species of monkeys were used, *Cebus capucinus* and *Macacus syrichtus*. All were normal healthy animals as far as could be determined by preliminary observation. No procedures to lower resistance or to injure the respiratory tract were resorted to before inoculation. It is well recognized[15] that the use of monkeys for experimental purposes requires great care in the interpretation of febrile reactions and of variations in the leucocyte count as evidences of infection. To establish the production of disease in the monkey the summation of clinical symptoms, temperature, and leucocyte reactions and the results of bacteriological and pathological examinations are essential. This is particularly true in the case of inoculations with *Bacillus influenzæ*, since it is clearly necessary, in view of the fairly well established toxic effects of influenza bacillus cultures and culture filtrates, [16,17] to show that the results obtained are due to actual infection with *Bacillus influenzæ* and are not merely the result of a temporary intoxication. The methods of study after inoculation, therefore, were the careful observation and recording of clinical symptoms, the taking of morning and evening rectal temperatures, daily total and differential counts of the leucocytes, microscopic examination of stained films of the respiratory tract secretions, and the taking of cultures of the nasal secretion and blood at irregular intervals. Since the disease produced did not prove fatal, most of the animals were killed by a sharp blow on the skull either during the acute stage of the disease or shortly after apparent recovery, and autopsies with bacteriological examination were immediately performed. Whenever

[15] Draper, G., and Hanford, J. M., *J. Exp. Med.*, 1913, xvii, 517.

[16] Parker, J. T., *J. Immunol.*, 1919, iv, 331.

[17] Huntoon, F. M., and Hannum, S., *J. Immunol.*, 1919, iv, 167.

Bacillus influenzæ was recovered in cultures during life or at autopsy it was identified as such by appropriate cultural tests.

Because of the extraordinary rapidity with which *Bacillus influenzæ* may lose its virulence when subcultured on artificial media outside the animal body, a particular effort was made to inoculate the monkeys with cultures as recently removed as possible from the passage monkeys. The material used for inoculation consisted of (1) first or second subculture on brown blood agar plates or in blood broth of *Bacillus influenzæ* recovered from the peritoneal exudate of monkeys of the passage series described above; (2) original or first subculture of *Bacillus influenzæ* recovered from the respiratory tract of a monkey (No. 138) killed during the acute stage of the pneumonia produced by intratracheal injection of the organism; or (3) the peritoneal exudate of monkeys of the passage series dying of *Bacillus influenzæ* peritonitis and septicemia. Comparatively young cultures—10 to 16 hours incubation—were used for inoculation. When plate cultures were employed the bacteria were washed off and suspended in sterile broth so that 1 cc. was equivalent to the growth on one plate. All cultures used for inoculation were tested for purity and identified as *Bacillus influenzæ.* In using peritoneal exudate directly the absence of bacterial contamination was controlled by microscopic examination of stained films before inoculation, and the material was subsequently shown by culture to contain only *Bacillus influenzæ.* The exudate was inoculated at varying intervals within an hour after removal at autopsy, being kept meanwhile at 37°C.

Two methods of inoculation were employed with different purposes in view. In one series of monkeys in order to determine whether *Bacillus influenzæ* would initiate an infection of the upper respiratory tract and, if so, what the characteristics of the disease produced might be, the material to be inoculated was introduced into the nose and mouth either by application to the mucous membranes with a cotton swab previously dipped in the culture material or by instillation with a pipette. In a second series of monkeys the material was introduced into the lower respiratory tract by direct intratracheal injection according to the method previously described.[2] The purpose of this series of experiments was to determine whether pneumonia could be experimentally produced in monkeys with *Bacillus influenzæ*, and, if

so, whether it would present the pathologic characteristics that have been ascribed to pure *Bacillus influenzæ* pneumonia in man by Pfeiffer,[5] MacCallum,[6] Wolbach,[7] and others.

EXPERIMENTAL.

Effect of Inoculating Monkeys on the Mucous Membranes of the Upper Respiratory Tract with Bacillus influenzæ.

Twelve monkeys received inoculations of *Bacillus influenzæ* on the mucous membranes of the nose or nose and mouth (Table II). The amount of culture or peritoneal exudate used in this series of experiments varied from what could be introduced by a single application in each nostril and the mouth of a cotton swab previously dipped in the material to be inoculated, up to 1 cc. of a blood broth culture of *Bacillus influenzæ* instilled into each nostril and 1 cc. into the mouth by a pipette. In every instance there resulted an acute respiratory infection which appeared to be essentially similar to influenza in man.

Clinical Course and Symptoms.—The onset occurred quite suddenly from 3 to 5 hours after inoculation. The first symptom was a variable degree of prostration, often extreme, the animals in many cases lying flat on the floor of the cage, eyes closed and very stuporous, sometimes pressing their hands tightly on the top of the head. This picture was in striking contrast with the onset of pneumococcus or streptococcus pneumonia in monkeys, for in these diseases prostration at onset was never observed, and it was frequently difficult to recognize by inspection that a monkey had become sick except for the accelerated respiration. Symptoms of upper respiratory tract infection soon followed. Frequent sneezing, blinking of the eyes, and rubbing of the nose were the prominent manifestations.

The subsequent course of the disease, though showing some variation in different monkeys, particularly with respect to the development of complications, was in general that of a self-limited infection, the acute stage of which lasted from 3 to 5 days. By the end of 24 hours a variable amount of nasal secretion had appeared, at first mucoid and scanty, later in some instances becoming more profuse and mucopurulent. Stained films and cultures of this secretion in some cases failed to show *Bacillus influenzæ* or at most showed only small num-

TABLE II.

Effect of Inoculating Monkeys on the Mucous Membranes of the Upper Respiratory Tract with B. influenzæ.

Monkey No.	Species.	Weight.	Date of inoculation.*	Material inoculated.	Strain of *B. influenzæ* inoculated.	Amount inoculated.		Result.	Complications.	*B. influenzæ* recovered at autopsy.	
						Nose.	Mouth.			U. R. T.	L. R. T.
		gm.	*1919*			*cc.*	*cc.*				
150	*C. capucinus.*	1,350	Aug. 25	10 hr. blood broth culture.	B. 11.11^{2}*	2.0	1.0	Acute respiratory disease. Improving on 4th day; killed.		−	+
154	" "	1,740	" 26	Peritoneal exudate of Monkey P 13b.	" 11.13^{0}	Swab.	Swab.	Acute respiratory disease. Improving on 4th day; killed.			+
153	" "	950	" 26	" "	" 11.13^{0}	1.0		Acute respiratory disease. Killed on 4th day.	Acute sinusitis, left antrum.	−	−
140	" "	1,600	" 26	" "	" 11.13^{0}	Swab.	Swab.	Acute respiratory disease. Improving on 4th day; killed.		−	+
148	" "	2,160	" 26	" "	" 11.13^{0}	"	"	Acute respiratory disease. Killed on 3rd day.		+	−
142	" "	1,300	" 26	" "	" 11.13^{0}	0.5		Acute respiratory disease. Killed on 4th day.	Acute sinusitis, left antrum; bronchopneumonia, R. L.	−	−
139	" "	1,405	" 26	" "	" 11.13^{0}	0.5		Acute respiratory disease. Killed on 3rd day.	Acute sinusitis, both antra.	+	−

157	*M. syrichtus.*	5,200	Aug. 26	Peritoneal exudate of Monkey P 13b.	B. 11.13[0]	2.0	1.0	Acute respiratory disease. Killed on 4th day.	Acute sinusitis, both antra. Bronchiolitis; bronchopneumonia, L. U., R. U., R. L.	+	+
158	" "	5,675	" 27	15 hr. blood broth culture.	" 11.13[3]	2.0	1.0	Acute respiratory disease. Recovered by 6th day.		No autopsy.	
159	" "	5,430	" 27	" "	" 11.13[3]	2.0	1.0	Acute respiratory disease. Killed on 6th day.	Acute sinusitis, both antra; bronchiolitis; bronchopneumonia, R. U., L. U., L. M., L. L.	+	+
160	*C. capucinus.*	1,500	" 27	" "	" 11.13[3]	1.5	1.0	Acute respiratory disease. Recovered by 6th day.		No autopsy.	
161	" "	1,340	" 27	" "	" 11.13[3]	1.5	1.0	Acute respiratory disease. Recovered by 6th day.		"	"

* This culture was a strain of *B. influenzæ* recovered at autopsy from Monkey 138 (Table III).

R. L., R. U., etc., indicate lobes of the lungs. U. R. T. indicates upper respiratory tract, including the antra; L. R. T., lower respiratory tract.

bers phagocytosed by polymorphonuclear leucocytes; in other cases *Bacillus influenzæ* was present in immense numbers. By 24 to 48 hours after inoculation the infection had spread to the lower respiratory tract, as evidenced by the development of a racking cough which suggested an inflammatory irritation of the trachea and bronchi. This symptom was striking in comparison with the cough of monkeys with pneumococcus pneumonia in which coughing occurred only late in the disease and was comparatively infrequent. That it was due to actual infection of the trachea by *Bacillus influenzæ* was shown by the recovery of the influenza bacillus from the trachea at autopsy, either in pure culture or in association with bacteria that are normally present in the mouths of monkeys.

The febrile reaction generally was not great. Four animals showed no elevation throughout the course of the disease. The character of the temperature curve was inconstant and bore no apparent relation to the severity of the infection, the afebrile animals being as sick as those that showed elevation of temperature. One fairly constant feature was a fall in temperature on the 2nd or 3rd day followed by a secondary rise which appeared to be associated with the development of tracheobronchitis.

The leucocyte counts during the active stage of the disease showed either a definite degree of leucopenia or no significant variation from the normal. The differential counts showed a very brief preliminary polymorphonuclear percentage increase followed by a fall to normal or subnormal level. In two cases a leucocytosis developed on the 2nd and 4th days respectively, autopsies in these instances showing a complicating purulent sinusitis.

Complications.—An acute purulent sinusitis affecting one or both antra developed in five monkeys. At autopsy the antra were found to contain a purulent exudate and the mucous membranes were edematous and congested. Cultures from the antra showed abundant influenza bacilli in three cases, twice in pure culture. In the other two none was recovered, although in one small numbers were seen in stained films of the exudate. In three instances other bacteria which are normal inhabitants of the upper respiratory tract of monkeys were also present.

Two monkeys (Nos. 157 and 159) developed a clinically recognizable bronchopneumonia on the 3rd and 4th days respectively. The onset of pneumonia was insidious and was suspected only because of a rise in temperature and a moderate acceleration of the respiratory rate. Both animals were killed early in the course of the pneumonia and *Bacillus influenzæ* was recovered in pure culture from the lungs. A third monkey (No. 142) developed a small patch of bronchopneumonia which was not recognized during life. The pathology of the disease and its complications are described in the following paper.[18]

Experiment 1.—Monkey 150 (Text-fig. 1, *a*). *Cebus capucinus*, male; weight 1,350 gm. Aug. 23 and 24, 1919. Well and active. Aug. 25, 10.30 a.m. 1 cc. of 10 hour blood broth culture of *B. influenzæ* (Strain B. 11.11^2) instilled into each nostril and 1 cc. into mouth with pipette. 2 p.m. Appears very sick and stuporous, lying curled up in corner of cage, paying little attention to surroundings; eyes dull; blinks lids frequently; presses hands on top of head; sneezes occasionally. 4.30 p.m. Condition the same; blood culture, no growth. 8 p.m. Sneezes very frequently. Aug. 26, 11 a.m. Still sick but less prostrated; refuses food; sneezes frequently; moderate mucoid nasal discharge; throat injected; stained film of nasal secretion shows many *B. influenzæ*, mostly phagocytosed; throat culture, *B. influenzæ*, non-hemolytic streptococci. 8 p.m. Condition the same. Aug. 27. Still sick, but appears better; sneezes occasionally; coughs occasionally; respirations normal. Aug. 28. Improving; sneezes and coughs occasionally. 10 a.m. Killed.

Autopsy. Anatomical Diagnosis.—Acute rhinitis; acute tracheobronchitis; hyperplasia of thymus.

Cultures.—Heart's blood, no growth; nasal mucous membranes, *Staphylococcus albus*; trachea, *B. influenzæ* and *Streptococcus viridans*.

Experiment 2.—Monkey 154 (Text-fig. 1, *b*). *Cebus capucinus*, male; weight 1,740 gm. Aug. 25, 1919. Well and active. Aug. 26, 11.15 a.m. Mucous membranes of nose and throat swabbed with cotton applicator previously dipped in salt solution washings from peritoneal cavity of Passage Monkey 13b (Strain B. 11.13^0). 1.30 p.m. Quiet; head drooping; eyes closed; sneezes occasionally. 4 p.m. Refuses food. 8 p.m. Sneezes very frequently. Aug. 27, 10 a.m. Quiet; sneezes very frequently. Moderate mucopurulent nasal discharge; stained film shows many polymorphonuclear leucocytes, few *B. influenzæ*, and Gram-positive cocci. 8 p.m. Condition the same; shakes head and rubs nose frequently. Aug. 28. Appears better; sneezes frequently and coughs occasionally. Aug. 29. Appears weak and exhausted; coughs frequently. Killed.

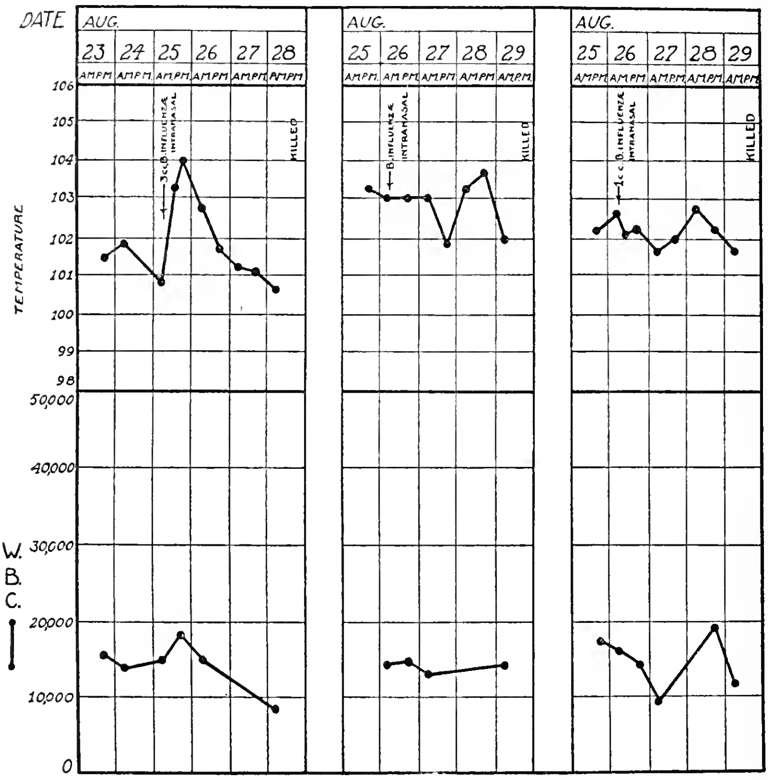
Autopsy. Anatomical Diagnosis.—Acute rhinitis; acute tracheobronchitis; hyperplasia of thymus.

---

[18] Cecil, R. L., and Blake, F. G., *J. Exp. Med.*, 1920, xxxii, 719.

Cultures.—Heart's blood, no growth; trachea, *B. influenzæ, Streptococcus viridans*, and Gram-negative micrococcus.

Experiment 3.—Monkey 153 (Text-fig. 1, *c*). *Cebus capucinus*, female; weight 950 gm. Aug. 25, 1919. Well and active. Aug. 26, 11 a.m. 0.5 cc. of salt solution washings from peritoneal cavity of Passage Monkey 13b (Strain B. 11.13[0])



a b c

Text-Fig. 1, *a* to *c*. Acute respiratory disease produced by inoculation of the mucous membranes of the upper respiratory tract with *B. influenzæ*. (*a*) Monkey 150. (*b*) Monkey 154. (*c*) Monkey 153; complicated by sinusitis.

instilled into each nostril with pipette. 1.30 p.m. Appears stuporous; head drooping. 4 p.m. Very sick; lying prostrate on floor of cage; refuses food. 8 p.m. Sneezes occasionally. Aug. 27. Still sick but appears better; sneezes frequently. Moderate mucoid nasal discharge; stained film shows moderate number of polymorphonuclear leucocytes, lymphocytes, and epithelial cells but

no bacteria. Aug. 28. Quiet; head drooping; eyes closed much of time; sneezes frequently. Aug. 29. Still sick; no animation; sneezes and coughs occasionally. Killed.

Autopsy. Anatomical Diagnosis.—Acute rhinitis; acute purulent sinusitis, left antrum; acute tracheobronchitis; hyperplasia of thymus.

Stained Films.—Tracheal exudate, many leucocytes and epithelial cells, few *B. influenzæ*; left antrum, abundant leucocytes, few *B. influenzæ*.

Cultures.—Heart's blood, trachea, and left antrum, no growth.

In the following experiment the effect of exposure to cold and wet on the subsequent course of the disease was studied. Monkeys 148 and 139 were exposed to cold and wet for 4 hours after inoculation by sprinkling them with cold water from time to time and by directing a current of air on them with an electric fan. Monkeys 140 and 142, not subjected to exposure, served as controls.

Experiment 4.—Monkey 140 (Text-fig. 2, *a*). *Cebus capucinus*, female; weight 1,600 gm. Aug. 23 to 25, 1919. Well and active. Aug. 26, 10.10 a.m. Mucous membranes of nose and throat swabbed with cotton applicator previously dipped in peritoneal exudate of Passage Monkey 13b (Strain B. 11.13^0). 1.30 p.m. Very quiet. 4 p.m. Quiet; head drooping; sneezes occasionally. Aug. 27. Moderately sick; refuses food; sneezes occasionally. Scanty mucoid nasal discharge; stained film shows leucocytes and epithelial cells but no bacteria; culture, no *B. influenzæ*. Aug. 28. Appears sicker; lying down; very drowsy; coughs and sneezes frequently. Aug. 29. Appears better but is quiet and exhausted; sneezes occasionally; coughs frequently. Killed.

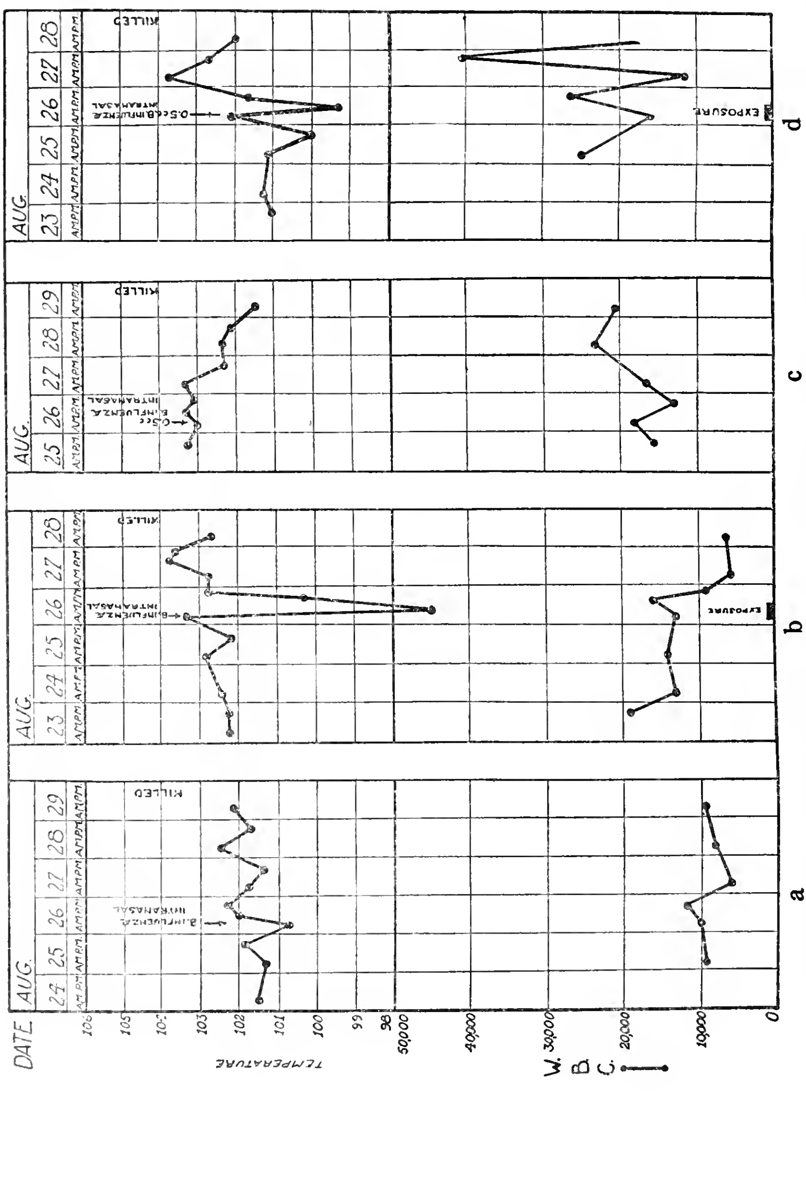
Autopsy. Anatomical Diagnosis.—Acute rhinitis; acute tracheobronchitis.

Cultures.—Heart's blood, no growth; nasal mucous membranes, *Staphylococcus albus* and large Gram-negative bacillus; trachea, *B. influenzæ, Staphylococcus albus*, and *Streptococcus viridans*.

Monkey 148 (Text-fig. 2, *b*). *Cebus capucinus*, female; weight 2,160 gm. Aug. 23 to 25, 1919. Well and active. Aug. 26, 10.20 a.m. Mucous membranes of nose and throat swabbed with cotton applicator previously dipped in peritoneal exudate of Passage Monkey 13b (Strain B. 11.13^0). 11 a.m. to 3 p.m. Exposure to cold and wet. 1.30 p.m. Very sick and stuporous, head drooping; eyes closed; shivering. 4 p.m. Prostrated; offers no resistance on handling; refuses food. Aug. 27. Very sick and prostrated; sneezes occasionally. Mucopurulent nasal secretion shows many leucocytes and epithelial cells and a few phagocytosed *B. influenzæ*. Aug. 28. Very sick; lying prostrate; sneezes frequently; no cough. Killed.

Autopsy. Anatomical Diagnosis.—Acute rhinitis; hyperplasia of thymus.

Cultures.—Heart's blood, trachea, and lungs, no growth; nasal mucous membranes, abundant *B. influenzæ* and few *Staphylococcus albus*.



TEXT-FIG. 2, *a* to *d*. Acute respiratory disease produced by inoculation of the mucous membranes of the upper respiratory tract with *B. influenzæ*. (*a*) Monkey 140. (*b*) Monkey 148. (*c*) Monkey 142; complicated by sinusitis and bronchopneumonia. (*d*) Monkey 139; complicated by sinusitis.

Monkey 142 (Text-fig. 2, *c*). *Cebus capucinus*, female; weight 1,300 gm. Aug. 23 to 25, 1919. Well and active. Aug. 26, 10.30 a.m. 0.25 cc. of peritoneal exudate from Passage Monkey 13b (Strain B. 11.13^0) instilled into each nostril with pipette. 1.30 p.m. Quiet; curled up in corner of cage; head drooping; eyes closed. 4 p.m. Quite sick and stuporous; refuses food; sneezes frequently. Aug. 27. Moderately sick; sneezes frequently. Mucopurulent nasal discharge shows many leucocytes and epithelial cells; no bacteria in stained film. Aug. 28. Still sick; sneezes very frequently. Aug. 29. Appears weak and without animation; sneezes occasionally. Killed.

Autopsy. Anatomical Diagnosis.—Acute rhinitis; acute sinusitis, left antrum; bronchopneumonia, right lower lobe.

Cultures.—Heart's blood and trachea, no growth; left antrum, few *Streptococcus viridans*.

Monkey 139 (Text-fig. 2, *d*). *Cebus capucinus*, female; weight 1,405 gm. Aug. 23 to 25, 1919. Well and active. Aug. 26, 10.40 a.m. 0.25 cc. of peritoneal exudate from Passage Monkey 13b (Strain B. 11.13^0) instilled into each nostril with pipette. 11 a.m. to 3 p.m. Exposure to cold and wet. 4 p.m. Very sick; head drooping; stuporous; eyes closed; shivering; refuses food. Aug. 27. Very sick; lying down; sneezes occasionally. Profuse mucopurulent nasal discharge showing abundant leucocytes, many *B. influenzæ*, and few staphylococci; culture, *B. influenzæ* and *Staphylococcus albus*. Aug. 28. Quite sick and stuporous; sneezes frequently and rubs nose; mucopurulent nasal discharge continues. 1.30 p.m. Killed.

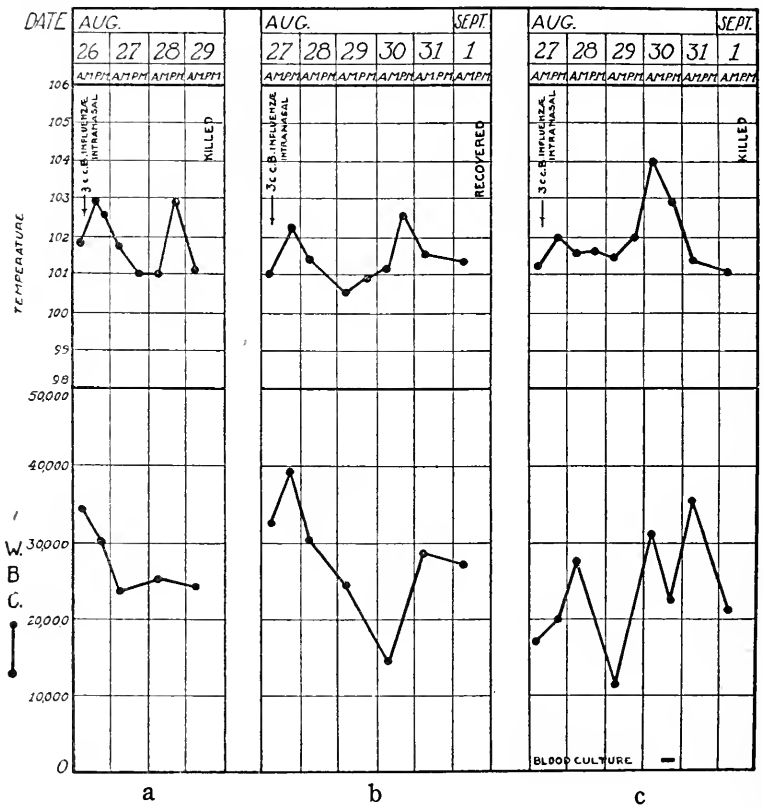
Autopsy. Anatomical Diagnosis.—Acute rhinitis; acute purulent sinusitis, both antra; acute tracheobronchitis; hyperplasia of thymus.

Cultures.—Heart's blood and trachea, no growth; both antra, abundant *B. influenzæ.*

Experiment 5.—Monkey 157 (Text-fig. 3, *a*). *Macacus syrichtus*, male; weight 5,200 gm. Aug. 25, 1919. Well, active, and ferocious. Aug. 26, 11.25 a.m. 1 cc. of salt solution washings of peritoneal cavity of Passage Monkey 13b (Strain B. 11.13^0) instilled into each nostril and 1 cc. into mouth. 2.30 p.m. Quite sick; lying down; stuporous; eyes closed. 4 p.m. Very sick; head drooping; refuses food. 10 p.m. Vomited. Aug. 27. Sick, no fight. Moderate mucopurulent nasal discharge; stained films show many polymorphonuclear leucocytes, many of them containing from two to twelve phagocytosed *B. influenzæ*; culture, no *B. influenzæ*. 2 p.m. Coughing violently; respirations not accelerated. Aug. 28. Still sick; sitting huddled up in corner of cage paying no attention to surroundings; coughs frequently; respirations moderately accelerated. Aug. 29. No animation; respirations accelerated; frequent cough. 11 a.m. Killed.

Autopsy. Anatomical Diagnosis.—Acute rhinitis; acute bilateral sinusitis of antra; acute tracheobronchitis; hemorrhagic edema of lungs; bronchiolitis; bronchiectasis; bronchopneumonia, left upper and right upper and lower lobes; hyperplasia of thymus.

Cultures.—Heart's blood and trachea, no growth; nasal mucous membrane, *B. influenzæ* and *Staphylococcus albus;* right antrum, *B. influenzæ* and Gram-negative micrococcus; right and left bronchi and right lower and upper lobes, pure culture of *B. influenzæ*; left upper lobe, *Streptococcus viridans.*



TEXT-FIG. 3, *a* to *c*. Acute respiratory disease produced by inoculation of the mucous membranes of the upper respiratory tract with *B. influenzæ*. (*a*) Monkey 157; complicated by sinusitis and bronchopneumonia. (*b*) Monkey 158. (*c*) Monkey 159; complicated by sinusitis and bronchopneumonia.

Experiment 6.—Monkey 158 (Text-fig. 3, *b*). *Macacus syrichtus*, male; weight 5,675 gm. Aug. 26, 1919. Well and ferocious. Aug. 27, 11 a.m. 3 cc. of 15 hour blood broth culture of *B. influenzæ* (Strain B. 11.13[3]) instilled into nose and mouth with pipette. 4 p.m. Quiet. Aug. 28. Appears moderately sick but without definite symptoms. Aug. 29 and 30. Condition the same; has developed a tight racking cough. Aug. 31. More active; coughs. Sept. 1. Appears well and active, but coughs occasionally. Observation discontinued.

Experiment 7.—Monkey 159 (Text-fig. 3, *c*). *Macacus syrichtus*, male; weight 5,430 gm. Aug. 26, 1919. Well, active, and ferocious. Aug. 27, 11.15 a.m. 3 cc. of 15 hour blood broth culture of *B. influenzæ* (Strain B. 11.13[3]) instilled into nose and mouth with pipette. 2 p.m. Appears sick; resting against side of cage. Aug. 28. Quiet. Mucoid nasal discharge; stained films show many leucocytes, some containing two to four *B. influenzæ*. Aug. 29. Has developed a dry racking cough; nasal discharge continues. 5 p.m. Appears quite sick; no animation; leaning against side of cage. Aug. 30 and 31. Condition the same; rubs eyes and nose and sneezes occasionally; coughs frequently; respirations accelerated. Sept. 1. Sick and exhausted; breathing accelerated; sneezes and coughs. 10.30 a.m. Killed.

Autopsy. Anatomical Diagnosis.—Acute rhinitis; acute bilateral sinusitis of antra; acute tracheobronchitis; hemorrhagic edema of lungs; bronchiolitis; bronchopneumonia, right upper and left upper, middle, and lower lobes; hyperplasia of thymus.

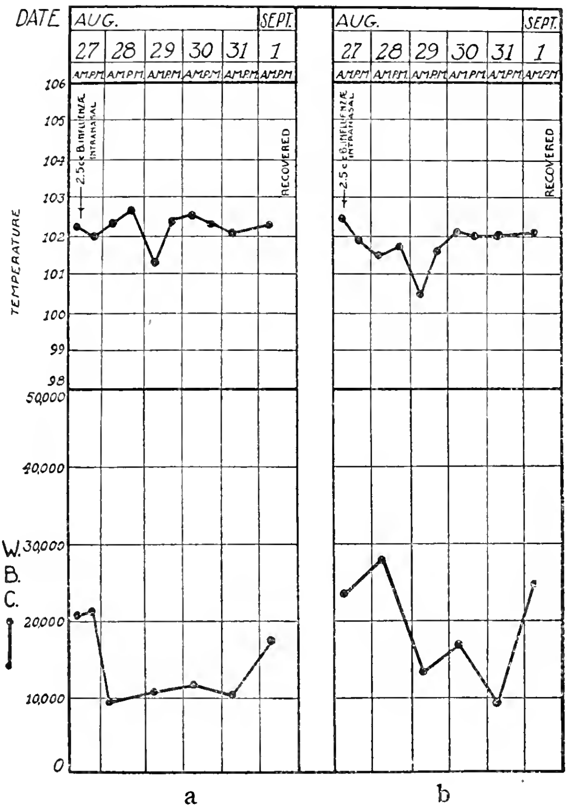
Cultures.—Heart's blood and left middle lobe, no growth; right antrum, pure culture of *B. influenzæ*; left antrum, *B. influenzæ* and *Streptococcus viridans*; trachea, *B. influenzæ*, *Streptococcus viridans*, and Gram-negative micrococcus; right and left bronchi, *B. influenzæ* and Gram-negative micrococcus; left lower and upper lobes, pure culture of *B. influenzæ*.

Experiment 8.—Monkey 160 (Text-fig. 4, *a*). *Cebus capucinus*, male; weight 1,500 gm. Aug. 26, 1919. Well and active. Aug. 27, 11.25 a.m. 2.5 cc. of 15 hour blood broth culture of *B. influenzæ* (Strain B. 11.13[3]) instilled into nose and mouth with pipette. 3 p.m. Sick; lying flat; rubs eyes; sneezes frequently. Aug. 28. Sick but less prostrated; sneezes and rubs eyes and nose frequently. Mucoid nasal discharge; stained film shows leucocytes and epithelial cells but no bacteria. Aug. 29. Condition the same. 3 p.m. Has begun to cough. Aug. 31. Appears better; sneezes and coughs occasionally. Sept. 1. Appears well. Observation discontinued.

Experiment 9.—Monkey 161 (Text-fig. 4, *b*). *Cebus capucinus*, male; weight 1,340 gm. Aug. 26, 1919. Well and active. Aug. 27, 11.35 a.m. 2.5 cc. of 15 hour blood broth culture of *B. influenzæ* (Strain B. 11.13[3]) instilled into nose and mouth with pipette. 1 p.m. Begins to appear sick. 2 p.m. Very sick; lying prostrate. 4 p.m. Lying down; stuporous; eyes closed; sneezes frequently. Aug. 28. Sick though less prostrated; mucopurulent nasal discharge; sneezes frequently. Aug. 29 and 30. Condition the same; coughs occasionally. Aug. 31. Appears better; coughs and sneezes occasionally. Sept. 1. Improving; observation discontinued.

In order to determine whether the culture medium in which *Bacillus influenzæ* had grown would exert any toxic effect when inoculated on the mucous membranes of the upper respiratory tract the following experiment was carried out.

Experiment 10.—Monkey 156. *Cebus capucinus*, female; weight 1,120 gm. Aug. 26, 1919. Well and active. 3 cc. of supernatant broth of a 16 hour blood broth culture of *B. influenzæ* (Strain B. 11.13[2]) freed from bacteria by centrifugalization instilled into nose and mouth with pipette. Monkey remained well and active, exhibiting no symptoms throughout a period of 6 days when observation was ended.



TEXT-FIG. 4, *a* and *b*. Acute respiratory disease produced by inoculation of the mucous membranes of the upper respiratory tract with *B. influenzæ*. (*a*) Monkey 160. (*b*) Monkey 161.

Effect of Intratracheal Injection of Bacillus influenzæ.

Ten monkeys were injected intratracheally with *Bacillus influenzæ* of enhanced virulence (Table III). These experiments may properly be divided into two groups of five animals each. In the first group

(Monkeys 124, 20, 137, 138, and 149) massive doses of *Bacillus influenzæ* were employed in an attempt further to increase the virulence of the organism for the respiratory tract of monkeys if possible. This did not prove feasible, however, and was abandoned. Three of these animals developed tracheobronchitis and bronchopneumonia, one bronchitis and bronchopneumonia, and one tracheobronchitis without pneumonia. In one instance (Monkey 138) *Bacillus influenzæ* pericarditis and septicemia ensued. Although all these animals were unquestionably infected with *Bacillus influenzæ* the value of the results is lessened because of the large doses employed. This is shown by the fact that a control monkey (No. 155) injected intratracheally with an equivalent amount of *Bacillus influenzæ* previously killed by heating at 56°C. for 1 hour, although showing none of the other evidences of actual infection exhibited by the monkeys inoculated with living cultures, did show moderate bronchopneumonic lesions at autopsy. In the second group (Monkeys 108, 136, 135, 141, and 152) a comparatively small dose (1 cc.) was employed with the successful production of bronchopneumonia in three animals. One developed tracheobronchitis without pneumonia and one resisted infection. In this group the development of pneumonia was clearly dependent upon actual infection with *Bacillus influenzæ*.

The course and general symptoms of the disease produced in these animals were similar to those occurring in the monkeys inoculated in the upper respiratory tract; that is, a self-limited respiratory disease of 3 to 6 days duration, sudden onset after a brief incubation period, prostration, a variable febrile reaction, and leucopenia, or no significant change in the leucocyte count. Leucocytosis with pericarditis occurred in Monkey 138. The disease was accompanied by a frequent and severe racking cough and accelerated respiratory rate. In no case did the disease prove fatal. It seems probable, however, that death would have occurred in the monkey that developed pericarditis and septicemia had it not been killed.

The nine monkeys showing clinical evidence of infection were killed at varying intervals after inoculation for pathological and bacteriological examination. In three of the seven monkeys which developed pneumonia *Bacillus influenzæ* was recovered in pure culture from the lungs or bronchi, in four the cultures remained sterile, indicating that

TABLE III.

Effect of Intratracheal Injection of B. influenzæ.

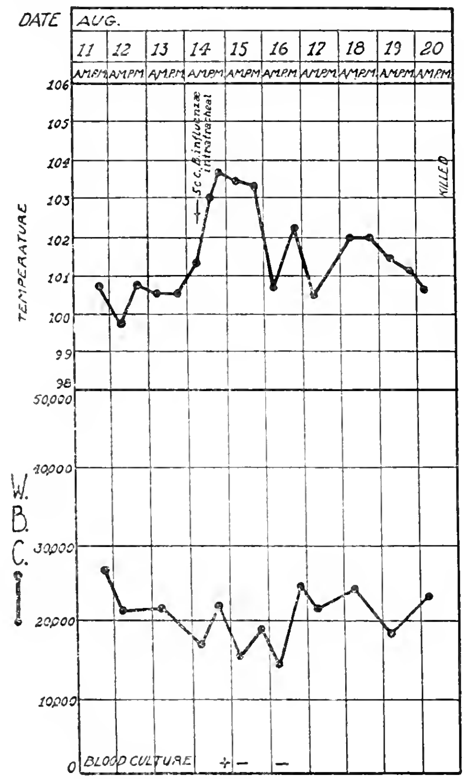
Monkey No.	Species.	Weight.	Date of injection.	Material injected.	Strain of *B. influenzæ* injected.	Amount injected.	Result.	Autopsy cultures.
		gm.	*1919*			*cc.*		
124	*M. syrichtus.*	1,500	Aug. 11	Broth suspension of 16 hr. plate culture.	B. 11.7[2]	5.0	Tracheobronchitis; bronchopneumonia. Killed on 3rd day.	H. B., n. g. Tr., B. I. Lungs, B. I.
20	" "	3,500	" 14	Broth suspension of 12 hr. plate culture.	" 11.8[2]	5.0	Tracheobronchitis. Recovered by 7th day. Killed.	H. B., n. g. Tr., B. I.
137	" "	2,830	" 19	" "	" 11.10[2]	4.0	Tracheobronchitis; bronchopneumonia. Killed on 3rd day.	H. B., n. g. Tr., " Lungs, "
138	" "	2,915	" 19	" "	" 11.10[2]	4.0	Tracheobronchitis; bronchopneumonia; acute pericarditis; septicemia. Killed on 3rd day.	H. B., B. I. Tr., " Lungs, " Pericard., "
149	*C. capucinus.*	1,500	" 23	" "	" 11.11[2]*	4.0	Bronchiolitis; bronchopneumonia. Recovered by 6th day. Killed.	H. B., n. g. Tr., " Lungs, "
108	*M. syrichtus.*	2,230	" 14	" "	" 11.8[2]	1.0	Tracheobronchitis. Recovered by 6th day. Killed.	H. B., n. g. Tr., "
136	*C. capucinus.*	1,100	" 14	Peritoneal exudate of Monkey P 8.	" 11.8[0]	1.0	Tracheobronchitis; hemorrhagic edema of lungs; bronchopneumonia. Killed on 6th day.	H. B., " Br., B. I. Lungs, n. g.
135	*M. syrichtus.*	2,150	" 14	Peritoneal exudate of Monkey P 9.	" 11.9[0]	1.0	No disease.	

141	*C. capucinus.*	1,400	Aug. 22	Broth suspension of 15 hr. plate culture.	B. 11.11^1*	1.0	Tracheobronchitis; bronchopneumonia; convalescent by 8th day. Killed.	H. B., n. g. Tr., " Turb., Staph. A.
152	" "	1,035	" 26	Peritoneal exudate of Monkey P 13b.	" 11.13^0	1.0	Tracheobronchitis; bronchopneumonia. Killed on 2nd day.	H. B., n. g. Tr., " Lungs, "

* This culture was a strain of *B. influenzæ* recovered from Monkey 138 at autopsy.

H. B. indicates heart's blood; Tr., trachea; Br., bronchi; turb., turbinates; B. I., *B. influenzæ*; n. g., no growth; Staph. A., *Staphylococcus albus*.

the infection had terminated. In the two animals in which only a tracheobronchitis developed, cultures from the trachea at autopsy yielded *Bacillus influenzæ* in pure culture in one, no growth in the other. Other bacteria, normal inhabitants of the upper respiratory tract of monkeys, were not found in this series. Their total absence



TEXT-FIG. 5. Monkey 20. Tracheobronchitis following intratracheal injection of *B. influenzæ*.

was in striking contrast with their frequent presence in the trachea in the series of monkeys inoculated on the mucous membranes of the upper respiratory tract in which it appeared that the spread of the influenza bacillus infection from the upper to the lower respiratory tract permitted the ready entrance of secondary organisms into the lower respiratory passages. Cultures of the heart's blood remained

sterile except in one instance in which *Bacillus influenzæ* was present in pure culture. The pathology of the disease is presented in the following paper.[18] Illustrative protocols follow.

Experiment 11.—Monkey 20 (Text-fig. 5). *Macacus syrichtus*, male; weight 3,500 gm. Aug. 11 to 13, 1919. Well and active. Aug. 14, 10.30 a.m. Intratracheal injection of 5 cc. of broth suspension (five plates) of 12 hour culture of *B. influenzæ* (Strain B. 11.8^2). 4.15 p.m. Appears quite sick; resting against side of cage; breathing rapidly. 9.30 p.m. Very sick; lying down. Aug. 15. Sick; lying down or leaning against side of cage most of time; respirations moderately accelerated; frequent hard, racking cough. Aug. 16. Condition the same; cough continues. Aug. 17 and 18. Improving, but still sick and without appetite; appears weak and exhausted; coughs frequently. Aug. 19. Appears better and more active; breathing easily; no cough. Aug. 20. Appears well. 9.40 a.m. Killed.

Autopsy. Anatomical Diagnosis.—Acute tracheobronchitis; hyperplasia of thymus and thoracic lymph nodes.

Cultures.—Heart's blood, no growth; trachea, pure culture of *B. influenzæ*.

Experiment 12.—Monkey 136 (Text-fig. 6, *a*). *Cebus capucinus* male; weight 1,100 gm. Aug. 14, 1919. Well and active. 2.30 p.m. Intratracheal injection of 1 cc. of peritoneal exudate of Passage Monkey 8, 10 minutes after removal of exudate at autopsy. 5 p.m. Begins to appear sick. 7 p.m. Very sick; lying prostrate; stuporous. 9 p.m. Prostrate; offers no resistance on handling; respirations accelerated. Aug. 15. Sick but no longer prostrated; no appetite; coughs. Aug. 16. Sick; lying down most of time; coughs occasionally. Aug. 17. Appears better but is weak and exhausted; no appetite; respirations moderately accelerated. Aug. 18. Condition the same. Aug. 19. More active. 11 a.m. Killed.

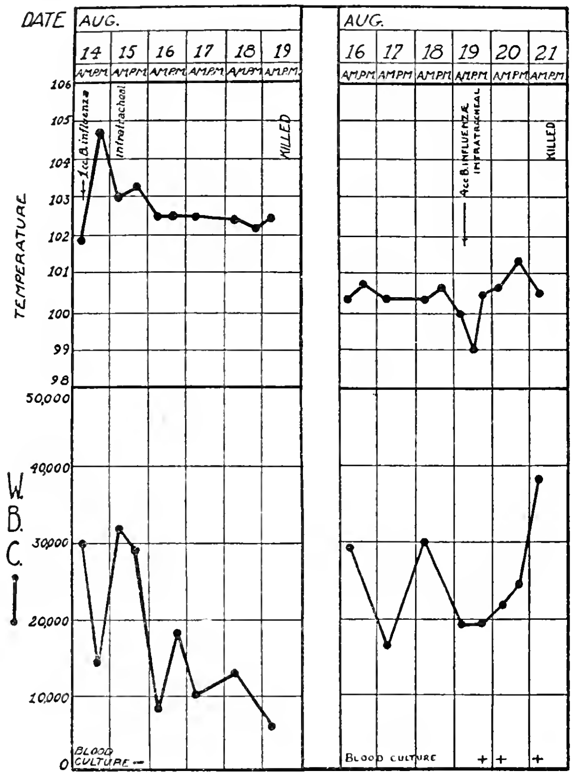
Autopsy. Anatomical Diagnosis.—Acute tracheobronchitis and bronchiolitis; hemorrhagic edema of lungs, all lobes; bronchopneumonia, all lobes; hyperplasia of thymus, spleen, and thoracic and abdominal lymph nodes.

Cultures.—Heart's blood and lungs, no growth; right bronchus, pure culture of *B. influenzæ*.

Experiment 13.—Monkey 138 (Text-fig. 6, *b*). *Macacus syrichtus*, female; weight 2,915 gm. Aug. 16 to 18, 1919. Well and active. Aug. 19, 10.45 a.m. Intratracheal injection of 4 cc. of broth suspension (four plates) of 12 hour culture of *B. influenzæ* (Strain B. 11.10^2). 4.30 p.m. Sick; resting against side of cage; respirations very rapid. 7.30 p.m. Lying prostrate; groaning; coughs occasionally. Aug. 20. Prostrated; breathing rapidly; coughs. Aug. 21. Very sick. 10 a.m. Killed.

Autopsy. Anatomical Diagnosis.—Acute tracheobronchitis; lobular bronchopneumonia, right lower, left upper, and left lower lobes; acute pericarditis; chronic fibrous pleuritis, right side; hyperplasia of thymus and spleen.

Cultures.—Heart's blood, trachea, left lower lobe, and pericardium, pure culture of *B. influenzæ*.



TEXT-FIG. 6, *a* and *b*. Effect of intratracheal injection of *B. influenzæ*. (*a*) Monkey 136; tracheobronchitis, hemorrhagic edema of lungs, and bronchopneumonia. (*b*) Monkey 138; tracheobronchitis, bronchopneumonia, pericarditis, and septicemia.

DISCUSSION AND SUMMARY.

Twelve normal monkeys inoculated on the mucous membranes of the nose or nose and mouth with a strain of *Bacillus influenzæ* originally isolated in pure culture from the pleural exudate of a case of empyema following influenzal pneumonia in man and subsequently raised in virulence by animal passage, developed an acute self-limited

respiratory disease of from 3 to 5 days duration, characterized by sudden onset with profound prostration, the development of rhinitis and tracheobronchitis, with sneezing, cough, and the outpouring of a scanty mucoid, or mucopurulent exudate, a variable febrile reaction, and either a leucopenia or no significant change in the leucocyte count. This disease was complicated in five instances by purulent sinusitis of one or both antra, in three by bronchopneumonia. *Bacillus influenzæ* was recovered at autopsy from the lesions of the disease either in pure culture or in association with organisms that are normal inhabitants of the upper respiratory tract of monkeys.

Of ten normal monkeys injected intratracheally with the same strain of *Bacillus influenzæ*, seven developed bronchopneumonia, two developed tracheobronchitis without pneumonia, and one resisted infection. The general symptoms and duration of the disease were similar to those of the preceding group. There were a severe cough and accelerated respirations. *Bacillus influenzæ* was recovered in pure culture from the lungs, bronchi, or trachea in the animals killed during the active stage of the disease. It disappeared promptly from the respiratory tract with recovery.

The significance of the first series of experiments in which monkeys were inoculated in the upper respiratory tract is twofold. First, they establish the fact that *Bacillus influenzæ* can initiate in monkeys an acute infection of the normal mucous membranes of the upper respiratory tract; that is, it can act as a primary incitant of respiratory infection without the assistance of a preceding or concomitant contributing cause. In this respect it differs radically from the pneumococcus and *Streptococcus hæmolyticus*, since experiments previously reported[2, 4] have shown that neither of these organisms possesses the property of initiating an infection of the normal mucous membranes of the upper respiratory tract of monkeys, even though the strains used were incalculably more virulent for monkeys than the strain of *Bacillus influenzæ* used in the foregoing experiments. Secondly, the experiments show that *Bacillus influenzæ* infection of the mucous membranes of the upper respiratory tract may spread by continuity to the paranasal sinuses, setting up an acute sinusitis, that it spreads readily to the lower respiratory tract, producing a tracheobronchitis and permitting the ready invasion of secondary bacteria, and that it may

penetrate as far as the terminal bronchioles, alveolar ducts, atria, and alveoli, there setting up a bronchiolitis and true bronchopneumonia. In these respects it likewise differs radically from the pneumococcus and *Streptococcus hæmolyticus* which do not possess these pathogenic properties as previous experiments have shown.[2, 4]

The bearing of these facts on the possible etiologic relation of *Bacillus influenzæ* to influenza is important, since they show that *Bacillus influenzæ* possesses certain definite primary pathogenic properties which distinguish it and therefore separate it from the group of recognized secondary organisms in influenzal complications, of which the pneumococcus and the streptococcus are the most frequent. The possible etiologic relation of *Bacillus influenzæ* to influenza is further supported by the character of the respiratory disease that occurred in the monkeys. The sudden onset with profound prostration, the absence of leucocytosis or often a leucopenia, the congestion of the mucous membranes of the respiratory tract, the development on the 2nd or 3rd day of an irritative cough due to an inflammatory tracheitis or tracheobronchitis, the brief self-limited course of the infection, and the irregular febrile reactions are all characteristic of influenza. Many of these symptoms were in striking contrast with the symptoms and course of pneumococcus or streptococcus infections in monkeys in which there were no prostration at onset, invariable leucocytosis, and infrequent cough developing only late in the disease.

While all the above features of the disease produced in monkeys are characteristic of influenza in man, none are pathognomonic and, in fact, it is doubtful whether uncomplicated influenza possesses any pathognomonic features by which it may be diagnosed certainly in the absence of an epidemic. Even during epidemic times many respiratory infections arise which, though presumably influenza, it is impossible to diagnose as such with certainty. Nor does pathology help in this respect, since there would appear to be no established distinctive lesions of uncomplicated influenza in man, nor for that matter of the complications of influenza, apart from the complications which have been ascribed by Pfeiffer,[5] MacCallum,[6] Wolbach,[7] and others to infection with *Bacillus influenzæ* because of the association of *Bacillus influenzæ* in pure culture with these complications. For these reasons, although the disease produced in monkeys appears

to be essentially identical with influenza in man with respect to its clinical course and complications, it is impossible to determine certainly whether it is actually so. The experiments are advanced, therefore, as evidence in favor of the etiologic relation of *Bacillus influenzæ* to influenza, though they do not permit of a definite conclusion in this respect.

Their bearing upon the relation of *Bacillus influenzæ* to certain of the complications of influenza would appear to be reasonably conclusive. The recovery of *Bacillus influenzæ* in pure culture at autopsy from the antra, from the trachea and bronchi, and from the lungs in some of the animals developing sinusitis, bronchiolitis, and a characteristic type of bronchopneumonia confirms by animal experiment the etiologic relation of *Bacillus influenzæ* to these complications of influenza, which hitherto has rested solely upon the frequent association of the influenza bacillus with these lesions in man. The production of tracheobronchitis and the same type of bronchopneumonia by the intratracheal injection of *Bacillus influenzæ* in the second series of experiments serves as additional confirmation of this, but has no direct bearing on the etiologic relation of *Bacillus influenzæ* to uncomplicated influenza.

CONCLUSIONS.

1. *Bacillus influenzæ* can initiate in normal monkeys an acute infection of the upper respiratory tract which may be complicated by acute sinusitis, tracheobronchitis, hemorrhagic edema of the lungs, bronchiolitis, and bronchopneumonia.

2. This disease appears to be essentially identical with influenza with respect to its clinical course, symptoms, and complications.

3. The etiologic relation of *Bacillus influenzæ* to acute sinusitis, tracheobronchitis, bronchiolitis, and bronchopneumonia is established.

4. Although it seems reasonable to infer from the results of the experiments that *Bacillus influenzæ* is the specific cause of influenza, a definite conclusion is not permissible, since it is impossible to determine whether the respiratory disease produced in monkeys with *Bacillus influenzæ* is identical with influenza or merely similar to it. The experiments are advanced, therefore, as evidence in favor of the etiologic relation of *Bacillus influenzæ* to influenza.

STUDIES ON EXPERIMENTAL PNEUMONIA.

X. Pathology of Experimental Influenza and of Bacillus influenzæ Pneumonia in Monkeys.

By RUSSELL L. CECIL, M.D., and FRANCIS G. BLAKE, M.D.

(*From the Bacteriological Laboratory of the Army Medical School, Washington.*)

Plates 90 to 97.

(Received for publication, June 15, 1920.)

The observation has frequently been made that influenza, as it occurs in man, possesses no characteristic pathology. The disease usually manifests itself primarily as a profound intoxication accompanied by a catarrhal inflammation of the upper respiratory tract, which, when uncomplicated, is rarely fatal. So far as known, the inflammatory changes display no properties which differentiate them from those occurring with other types of infection in the same locality. Further confusion is added to the pathology of influenza by reason of the mixed bacteriological findings. While *Bacillus influenzæ* can usually be isolated from the secretions, it is generally found in conjunction with other bacteria, such as streptococcus or pneumococcus. This lack of distinctive features in the pathology of influenza has greatly enhanced the difficulty of identifying the disease in man and of studying it experimentally in animals. None of the earlier investigators made any claims to having transmitted influenza to animals.

Pfeiffer[1] carried out many experiments on various laboratory animals, including monkeys, injecting them intratracheally, intravenously, and directly into the lung tissue, but always without positive results. If large doses of culture were administered the animals usually showed symptoms of toxemia and sometimes died; but he was never able to demonstrate an actual multiplication of the influenza bacilli—a true infection.

In respect to the pathology of *B. influenzæ* pneumonia, accurate knowledge has been almost as difficult to obtain as in influenza. Wollstein and Meltzer[2]

---

[1] Pfeiffer, R., *Z. Hyg. u. Infectionskrankh.*, 1893, xiii, 357.

[2] Wollstein, M., and Meltzer, S. J., *J. Exp. Med.*, 1912, xvi, 126.

succeeded in producing patches of consolidation in the lungs of dogs by insufflating massive doses of influenza bacilli directly into the bronchi, but with this exception, *B. influenzæ* pneumonia has never been reproduced in animals; and pure *B. influenzæ* pneumonia in man is so rarely encountered that it has been hard to say which of the lesions are due to the influenza bacillus, and which to the other bacteria present. Although *B. influenzæ* pneumonia is usually characterized by the presence of other bacteria, notably the streptococcus and pneumococcus, in addition to the influenza bacillus, pure *B. influenzæ* infections occur, and several investigators have described the pathological picture in such cases (Pfeiffer,[1] MacCallum,[3] and Wolbach[4]).

The descriptions by these writers of the pathology of *B. influenzæ* pneumonia in man are quite similar and indicate clearly that pure *B. influenzæ* infections of the lung produce a characteristic group of lesions. Briefly, these authors describe the condition as follows: Dark red, partially air-containing lungs; slight if any pleural exudate; on section, edema and hemorrhage; purulent bronchitis and bronchiolitis; peribronchial infiltration and thickening; bronchopneumonia; bronchiectasis and emphysema. Microscopically serous and hemorrhagic exudate; dilatation of bronchioles and alveoli; partial destruction of bronchial mucous membrane; pus in bronchi and bronchioles; round cell infiltration and thickening of bronchial walls; infiltration of leucocytes in neighboring alveolar walls; leucocytes and desquamated epithelium in adjacent alveoli; deposit of hyaline material on alveolar walls; organization.

In the preceding article[5] it has been shown that by inoculating monkeys intranasally with a virulent culture of *Bacillus influenzæ*, an infection of the upper respiratory tract may be induced which is clinically comparable to influenza in man, and furthermore, that intratracheal injections of virulent influenza bacilli excite in monkeys a bronchopneumonia analogous to *Bacillus influenzæ* pneumonia in man. In the present paper the pathological findings of experimental *Bacillus influenzæ* respiratory infections will be discussed in detail.

As influenza in man is practically never a fatal disease unless complicated by pneumonia, there has been little or no opportunity for studying the general pathology of the disease. There is no reason to believe, however, that simple influenza is regularly accompanied by

[3] MacCallum, W. G., *J. Am. Med. Assn.*, 1919, lxxii, 720; The pathology of the pneumonia in the United States Army camps during the winter of 1917–18, Monograph of The Rockefeller Institute for Medical Research, No. 10, New York, 1919.

[4] Wolbach, S. B., *Bull. Johns Hopkins Hosp.*, 1919, xxx, 104.

[5] Blake, F. G., and Cecil, R. L., *J. Exp. Med.*, 1920, xxxii, 691.

extensive pathological changes in the various internal organs. It appears to be a local infection of the upper respiratory tract, the general symptoms of such infection probably being referable to absorption of toxic substances. Briefly, it may be said that uncomplicated influenza in man is characterized by swelling and hyperemia of the nasal and pharyngeal mucous membrane with a variable amount of mucoid discharge which later may become profuse and mucopurulent, and usually by acute inflammatory changes in the trachea and larger bronchi. Injection of the conjunctivæ and flushing of the face are also frequently seen. In uncomplicated cases there is little evidence pointing to significant pathological lesions of the internal organs.

Pathology of Experimental Influenza in Monkeys.

In the preceding paper the method of producing experimental influenza in monkeys has been described in detail, and will, therefore, not be repeated here.

In Table I the monkeys that were infected by the inoculation of virulent influenza bacilli into the nose and throat are presented, with the dose injected and the pathological findings in each. Three of these monkeys (Nos. 158, 160, and 161) were not killed. They presented, however, all the clinical signs of acute rhinitis and acute tracheitis. The remaining nine monkeys were killed during, or immediately following their infection, and were subjected at once to complete autopsy.

Rhinitis.—By referring to Table I it will be seen that at autopsy each of the nine monkeys showed acute rhinitis, and that in five out of the nine *Bacillus influenzæ* was recovered by culture or film from some part of the upper respiratory tract (Nos. 148, 139, 153, 157, and 159). The nasal mucous membrane in these nine monkeys presented very much the same appearance. The mucosa was swollen and intensely reddened, especially over the turbinates. The blood vessels were congested, and the mucous membrane was covered with a mucoid or mucopurulent exudate. In short, the process was analogous to acute rhinitis in man.

Microscopic sections from the nasal mucous membrane show an exudate of mucus, pus cells, and desquamated epithelial cells on the

TABLE I.

Monkeys That Received B. influenzæ in the Nose and Throat.

Monkey No.	Date of inoculation.	Amount inoculated.		Date killed.	Pathological findings.	Autopsy cultures.
		Nose.	Throat.			
	1919	*cc.*	*cc.*	*1919*		
150	Aug. 25	2.0	1.0	Aug. 28	Rhinitis; tracheobronchitis; hyperplasia of thymus.	Nasal m. m., Staph. A. Tr., B. I.; Str. V.
140	" 26	Swab.	Swab.	" 29	Rhinitis; tracheobronchitis.	Turb., Staph. A.; large Gr.-neg. bac. Tr., B. I; Staph. A.; Str. V.
148	" 26	"	"	" 28	" hyperplasia of thymus.	Turb., B. I.; Staph. A. Tr., n. g. Lungs, n. g.
142	" 26	0.5		" 29	" sinusitis of left antrum; bronchopneumonia, R. L.	Left antrum, Str. V. Tr., n. g.
139	" 26	0.5		" 28	Rhinitis; bilateral sinusitis; tracheobronchitis; hyperplasia of thymus.	Right antrum, B. I. pure. Left " " " Tr., n. g.
153	" 26	1.0		" 29	Rhinitis; tracheobronchitis; sinusitis of left antrum; hyperplasia of thymus and cervical lymph glands.	Left antrum, n. g.; few B. I. in films. Tr., n. g.; few B. I. in films.
154	" 26	Swab.	Swab.	" 29	Rhinitis; tracheobronchitis; hyperplasia of thymus and cervical lymph glands.	" B. I.; Str. V.; Gr.-neg. micr.

157	Aug. 26	2.0	1.0	Aug. 29	Rhinitis; tracheobronchitis; bilateral sinusitis of antra; hemorrhagic edema of lungs and bronchopneumonia, R. U., L. U., R. L.; hyperplasia of thymus and thoracic lymph glands; bronchiectasis; emphysema.	Left turb., B. I.; Staph. A. Right antrum, B. I.; Gr.-neg. coccus. Tr., n. g. Both br., pure B. I. R. U., pure B. I. R. L., " " L. U., no B. I.; 6 col. Str. V.
158	" 27	2.0	1.0	Not killed.	(Rhinitis; tracheitis.)	
159	" 27	2.0	1.0	Sept. 1	" tracheobronchitis; bilateral sinusitis of antra; hemorrhagic edema of lungs; bronchopneumonia, L. U., L. M., R. U.; organizing pneumonia, L. L.; hyperplasia of thymus and thoracic lymph glands.	Tr., B. I.; few Gr.-neg. micr. and Str. V. Br., B. I.; Gr.-neg. micr. Left antrum, B. I.; Str. V. Right " pure B. I. L. U., pure B. I. L. M., n. g. L. L., pure B. I.
160	" 27	1.5	1.0	Not killed.	(Rhinitis; tracheitis.)	
161	" 27	1.5	1.0	Not killed.	" "	

R. L., L. L., etc., indicate lobes of the lung.

Br. indicates bronchus; m.m., mucous membrane; turb., turbinates; n. g., no growth; Staph. A., *Staphylococcus albus*; B. I., *B. influenzæ*; Str. V., *Streptococcus viridans*; Gr.-neg., Gram-negative. The other abbreviations are self-explanatory.

surface of the epithelium, which is eroded in some places (Fig. 1). The submucosa is engorged, and is infiltrated with polymorphonuclear leucocytes, many of which are also seen between the columnar epithelial cells. Goblet cells are numerous.

Tracheobronchitis.—In seven of the nine animals (Nos. 150, 140, 139, 153, 154, 157, and 159) the inflammation had extended from the nasopharynx down into the trachea and bronchi. Tracheobronchitis can hardly be called a complication in this instance, since it occurs in monkeys, as in man, in a large percentage of cases. In this group the trachea and bronchi were sometimes reddened, but more often the mucous membrane showed only an injection of the blood vessels and a deposit of mucus over the surface. Cultures from the trachea or large bronchi yielded *Bacillus influenzæ* in five out of seven cases (Nos. 140, 150, 154, 157, and 159). Microscopic sections from the trachea show acute inflammatory changes similar to those found in the nasal mucous membrane (Figs. 2 and 3).

Sinusitis.—Five of the nine cases that were autopsied showed, in addition to rhinitis, an infection of one or both of the antra of Highmore (Nos. 142, 139, 153, 157, and 159). *Bacillus influenzæ* infection of the antrum in monkeys is similar to that in man. The mucous membrane is reddened and swollen and the cavity is partially filled with mucopurulent exudate which in one instance was tinged with blood. Cultures from the antra showed influenza bacilli in three cases (Nos. 139, 157, and 159). Of the remaining two, one revealed influenza bacilli in films (Monkey 153) and the other (Monkey 142) yielded *Streptococcus viridans*, probably a secondary invader that had supplanted the influenza bacillus.

Hyperplasia of the Lymph Glands.—The cervical lymph glands were enlarged in two monkeys (Nos. 153 and 154). Microscopic sections show hyperplasia of the follicles, with an increased number of leucocytes in the sinuses.

Hyperplasia of the Thymus.—One of the most interesting changes observed in these monkeys was a well marked hyperplasia of the thymus gland. This condition was entirely unexpected, and so far as known has not been described in *Bacillus influenzæ* infections in man. In seven of the nine monkeys that were autopsied (Monkeys 150, 148, 139, 153, 154, 157, and 159) the thymus gland was definitely

enlarged, in some instances extending down for a considerable distance over the pericardium. The gland was rather firm and had a peculiar pinkish yellow, flesh-like appearance. The lobules were readily recognized. Microscopic sections show marked hyperplasia of the follicles, due to proliferation of the large cells composing the chyme centers. The lymphatic channels leading to the gland are greatly dilated and filled with coagulated plasma. Even the small channels in the center of the follicles are often dilated, and, in addition, contain a considerable number of polymorphonuclear leucocytes and eosinophils. In some instances the interstitial tissue between the lobules is densely infiltrated with leucocytes.

The following case of experimental influenza illustrates the lesions above described and is reported in full.

Protocol 1.—Monkey 139. *Cebus capucinus*, female; weight 1,405 gm. Aug. 26, 1919. Injected with 0.5 cc. of *B. influenzæ* peritoneal exudate intranasally. Aug. 28. Killed by blow on cervical vertebræ.

Autopsy.—Subject is a small brown Capuchin. Thymus shows moderate enlargement. Pericardium and pleura normal; heart is not enlarged; myocardium and valves normal. Trachea and bronchi contain a moderate quantity of mucus. Bronchial lymph glands are not enlarged. Nasal cavities contain a considerable amount of mucopurulent material. Turbinates on both sides are swollen and reddened and their vessels stand out prominently. The right antrum contains 10 or 12 drops of cloudy fluid; the mucous membrane is swollen and edematous. The left antrum also contains cloudy fluid and is similar in appearance to the right. The lungs are pale and voluminous and free from consolidation. Abdominal organs present nothing of importance.

Cultures.—Right antrum, pure culture of *B. influenzæ*; left antrum, pure culture of *B. influenzæ*; trachea, no growth.

Anatomical Diagnosis.—Acute rhinitis; acute bilateral sinusitis; acute tracheobronchitis; hyperplasia of thymus.

Microscopic Examination.—*Trachea.*—Blood and mucus on surface of epithelium, which shows many goblet cells. Vessels of submucosa engorged; infiltration of leucocytes beneath epithelium. *Turbinate.*—Mucous membrane covered with a thick layer of exudate composed of red blood cells, leucocytes, and desquamated epithelial cells (Fig. 1). The epithelium is eroded in many places. Numerous leucocytes are working their way out between the epithelial cells. There is infiltration of leucocytes in the submucosa. Blood vessels of submucosa are distended with red blood cells. *Lungs.*—Normal. *Thymus.*—Shows hyperplasia of follicles and distention of lymph channels with coagulated plasma.

Three of the monkeys in this group developed bronchopneumonia (Nos. 142, 157, and 159), which is a complication of such importance that it will be discussed in considerable detail.

Pathology of Bacillus influenzæ Pneumonia in Monkeys.

Bacillus influenzæ Pneumonia Developing Spontaneously in Monkeys with Experimental Influenza.

In three of the twelve monkeys inoculated in the nose and throat with *Bacillus influenzæ*, the infection in the upper respiratory tract extended down into the bronchioles with the production of a bronchiolitis, peribronchiolitis, and bronchopneumonia.

Protocol 2.—Monkey 142. *Cebus capucinus*, female; weight 1,300 gm. Aug. 26, 1919. Received 0.5 cc. of *B. influenzæ* peritoneal exudate in the nasal cavities. Aug. 29. Killed.

Anatomical Diagnosis.—Acute rhinitis; acute sinusitis of left antrum; bronchopneumonia, right lower lobe.

Autopsy.—Subject is a small Capuchin; weight 1,300 gm. Lungs show no definite consolidation in the gross. Turbinates greatly swollen and reddened and covered with mucus. Left antrum contains blood-tinged cloudy fluid. Right antrum is clear.

Cultures.—Left antrum, *Streptococcus viridans*; trachea, no growth.

Microscopic Examination.—Turbinates.—Extensive infiltration of leucocytes in mucosa and submucosa. *Right lower lobe.*—Shows an acute bronchiolitis with early pneumonic changes. At certain points the epithelium of the bronchiole is absent and its place taken by a deposit of fibrin and leucocytes. The wall of the bronchiole is infiltrated with polymorphonuclear leucocytes and lymphoid cells and the infiltration extends into the alveolar walls of the adjacent tissue. The capillaries of the alveolar walls are markedly distended in this neighborhood, and a few red blood cells and leucocytes have escaped into the alveoli. The changes, however, are for the most part interstitial and sharply limited to the vicinity of the bronchioles. Section of the right lower lobe stained for bacteria shows no influenza bacilli or other bacteria.

This case illustrates *Bacillus influenzæ* pneumonia in the very earliest stage and indicates that the process is essentially peribronchiolar; in other words, a true bronchopneumonia. Unfortunately, the pneumonia was so early that it was not recognized at autopsy, so no cultures were taken from the lungs. There is every reason to believe, however, that *Bacillus influenzæ* was the exciting agent.

The second case presented a more extensive *Bacillus influenzæ* pneumonia. In this animal the process was readily recognized during life and at autopsy. The lungs showed the hemorrhagic edema and type of consolidation so frequently seen in *Bacillus influenzæ* pneumonia in man.

Protocol 3.—Monkey 157. *Macacus syrichtus*, male; weight 5,200 gm. Aug. 26, 1919. Received 2 cc. of *B. influenzæ* peritoneal exudate intranasally; also received 1 cc. of same peritoneal exudate in the throat. Aug. 29. Killed.

Anatomical Diagnosis.—Acute catarrhal rhinitis following instillation of *B. influenzæ*; bilateral sinusitis of antra of Highmore; acute tracheobronchitis and bronchiolitis; hemorrhagic edema of lungs; bronchopneumonia; vesicular emphysema; bronchiectasis; hyperplasia of thymus and thoracic lymph glands.

Autopsy.—Subject is a large male *Macacus.* Thymus moderately enlarged. A few old adhesions in both pleural cavities. The greater part of the right upper lobe is intensely congested and dark red in color. Emphysematous lobules stand out prominently over the engorged area. A small portion of the upper posterior part of the right lower lobe shows a similar engorgement and emphysema. There is a small patch of engorgement at the root of the right middle lobe. The cut surface of the involved portion of the right lung is dark red and oozes a considerable quantity of bloody fluid. The bronchi are very conspicuous on the cut surface and appear to be much distended. The left upper lobe is covered with old fibrous tags. There is a large area of engorgement along the posterior border similar to that in the right upper lobe. The bronchial lymph glands are greatly swollen. The mucous membrane of the trachea and bronchi is congested and covered with blood-tinged mucus. Liver and kidneys show slight cloudy swelling. Other abdominal organs present nothing of importance. Testes normal. Rectus abdominis muscle appears normal. Nasal mucous membrane is reddened and covered with mucus. Turbinates, especially the left, are intensely swollen and engorged, and have a purplish tinge. Both antra contain pus.

Cultures.—Left turbinate, *B. influenzæ* and *Staphylococcus albus*; right antrum, *B. influenzæ* and Gram-negative coccus; trachea, no growth; both bronchi, pure culture of *B. influenzæ*; right upper lobe, pure culture of *B. influenzæ*; right lower lobe, pure culture of *B. influenzæ*; left upper lobe, six colonies of *Streptococcus viridans.*

Microscopic Examination.—*Turbinates.*—Marked swelling of the epithelial cells. There is a deposit of blood and mucus on the surface of the epithelium. A considerable number of leucocytes are found working their way between the epithelial cells and there is moderate infiltration of leucocytes in the submucosa. *Trachea.*—Deposit of mucus containing leucocytes on surface of the epithelium. Many goblet cells; a considerable number of polymorphonuclear leucocytes working their way out between the epithelial cells. *Right upper lobe.*—Shows pneumonic process in several phases. In some places there are marked edema and

engorgement (Fig. 4). In other places the alveoli are filled with serum and red blood corpuscles, while at still other points an exudate of leucocytes, fibrin, and desquamated epithelial cells fills the alveoli. The process is very irregular and peribronchiolar in distribution. There is a striking absence of the perivascular edema and infiltration of leucocytes so frequent in pneumococcus and streptococcus pneumonia. Another feature is the marked alveolar emphysema which is seen at many points (Figs. 4 and 5). The alveoli are much larger than normally and the alveolar walls are thin. In many places the great distention of the alveoli has resulted in rupture of the walls. The bronchioles contain blood and coagulated plasma and in some places a leucocytic exudate (Fig. 5). The walls of the bronchioles are infiltrated with leucocytes, and their epithelial lining has been destroyed in places. Some of the bronchi and bronchioles are greatly dilated (Fig. 6), and their epithelial cells show marked flattening. There are chronic inflammatory changes with organization about some of the blood vessels, but these appear to be the sequel of an old infection. There are a few minute intracellular Gram-negative bacilli, all in the leucocytes in the alveoli or bronchioles. *Right lower lobe.*—There is considerable interstitial infiltration of leucocytes in the neighborhood of the bronchioles, and in such areas the alveoli also contain exudate. Emphysema well marked. There are a few influenza bacilli in some of the alveoli. The bacteria are intracellular, having been phagocytosed by the leucocytes composing the alveolar exudate. *Left upper lobe.*—Considerable congestion, but no exudate. No influenza bacilli found. *Thymus gland.*—Well marked hyperplasia of the follicles. There is considerable infiltration of eosinophils, especially around the small central lymph channels. The larger lymphatic channels are greatly dilated, and are filled with coagulated plasma. *Spleen, testes, pancreas, and rectus abdominis muscle.*—Normal.

The third case of *Bacillus influenzæ* pneumonia following experimental influenza also presented characteristic changes.

Protocol 4.—Monkey 159. *Macacus syrichtus*, male; weight 5,430 gm. Aug 27, 1919. Received 1 cc. of blood broth culture of *B. influenzæ* in each nostril, and 1 cc. in the throat. Sept. 1. Killed.

Anatomical Diagnosis.—Acute rhinitis; acute tracheobronchitis; bilateral sinusitis of antra; hemorrhagic bronchopneumonia, left upper and middle lobes, and right upper lobe; lobar pneumonia, stage of resolution and organization, left lower lobe; hyperplasia of thymus and thoracic lymph glands.

Autopsy.—Subject is a large male *Macacus*. Thymus greatly enlarged, extending down to the middle of the pericardium. It is pinkish yellow and measures 3 inches in length. Pericardial sac normal; heart slightly dilated. The lungs are voluminous. The left upper and middle lobes show, extending outward from the hilum, deep red patches of engorgement and edematous consolidation. In these patches emphysematous air sacs are prominent. Cut surface shows irregular patches of consolidation, and exudes a large amount of bloody frothy

fluid. In the upper and posterior portion of the left lower lobe there is an area of moderately firm consolidation about 3 cm. in length. The cut surface of this area is pale yellowish gray, slightly moist, and has a translucent appearance (old organization). It is sharply defined from the unconsolidated portions of the lobe. The right upper lobe shows a few small patches similar to those in the left upper lobe. The remainder of the lungs is crepitant.

Cultures.—Trachea, *B. influenzæ* and a few Gram-negative micrococci and *Streptococcus viridans*; bronchi, *B. influenzæ* and Gram-negative micrococci; left antrum, *B. influenzæ* and *Streptococcus viridans*; right antrum, pure culture of *B. influenzæ*; left upper lobe, pure culture of *B. influenzæ*; left middle lobe, no growth; left lower lobe, pure culture of *B. influenzæ*.

Microscopic Examination.—*Left upper lobe.*—Extensive consolidation in one part of the section, which is apparently of some duration, as well advanced resolution and organization are present in many places. The exudate is found chiefly in the alveoli, and consists of lymphoid cells and polymorphonuclear leucocytes. Desquamated epithelial cells are also found in considerable numbers in the alveoli. Some of the alveoli contain plugs of organizing fibrin. There is a thick zone of lymphoid cells and connective tissue around the blood vessels. The lesions are those of a resolving lobar pneumonia. The whole lobe, however, is not implicated. Just beyond the consolidated area, the tissue shows engorgement and hemorrhage into the alveoli. A few influenza bacilli are seen in the bronchi. *Left middle lobe.*—Main bronchus filled with blood and leucocytes. In the neighborhood of the large vessels the lung tissue shows intense engorgement and hemorrhage with extravasation of red blood corpuscles into the alveoli and moderate edema. Pleura has been converted into a thick coat of organizing granulation tissue with a deposit of fibrin on the surface (result of a previous infection). Peripheral portion of section is free from consolidation. There are patches of compensatory emphysema in some places. There are large numbers of influenza bacilli in the bronchi, but none in the alveoli. *Left lower lobe.*—Sections show an extensive exudate in practically all the alveoli, resembling that seen in pneumococcus pneumonia. Resolution and organization are in process. Marked perivascular infiltration. Bronchi filled with pus. No bacteria found. *Trachea.*—Deposit of mucus on surface of mucous membrane; mucosa and submucosa infiltrated with leucocytes (Fig. 2). *Tracheal lymph gland.*—Hyperplasia of follicles and infiltration of considerable number of polymorphonuclear leucocytes in the sinuses. Some of the leucocytes are undergoing disintegration and phagocytosis by macrophage cells. *Turbinate.*—Mucosa for the most part intact, but the epithelial cells are swollen and filled with mucus. Heavy deposit of mucus and leucocytes on surface of mucosa. Leucocytes are seen working out between the epithelial cells, and the submucosa is densely infiltrated with polymorphonuclear leucocytes, lymphocytes, and plasma cells. Blood vessels distended with red blood corpuscles. *Thymus.*—Marked hyperplasia of the follicles, and distention of lymph channels which are filled with coagulated plasma. The small capillaries in the follicles are filled with leucocytes.

In Monkey 159 we have a further illustration of *Bacillus influenzæ* pneumonia, in this instance, however, apparently superimposed on an old unresolved pneumonia of the lobar type. Many of the alveoli contain only desquamated epithelial cells or plugs of fibrin, while others are filled with young connective tissue cells. Such areas resemble an organizing pneumococcus pneumonia. On the other hand, extensive areas of hemorrhage and edema occur, and in the latter places the picture is that of a *Bacillus influenzæ* infection.

These cases of *Bacillus influenzæ* pneumonia in monkeys present altogether a fairly complete reproduction of *Bacillus influenzæ* pneumonia as it occurs in man. Complete absence or scarcity of fibrin, hemorrhage and edema, bronchiolitis and peribronchiolar infiltration, discrete or confluent bronchopneumonia, and, finally, emphysema and bronchiectasis, are characteristic lesions which have been demonstrated in both man and monkey. In these three cases the infection originated in the nose and throat and extended down the bronchial tree to the terminal bronchioles, exciting in them a purulent bronchiolitis. Peribronchiolar infiltration with involvement of the adjacent alveoli followed (Fig. 5). The hemorrhage and edema in these animals probably represent a reaction on the part of the lung to absorbed toxic substances; the emphysema is partially compensatory, but for the most part is doubtless caused by the obstructing plugs of pus in the bronchioles.

Bacillus influenzæ Pneumonia in Monkeys Following Intratracheal Injection of Bacillus influenzæ.

Experimental *Bacillus influenzæ* pneumonia was produced by injecting virulent influenza bacilli directly into the trachea, as described in the preceding paper. In Table II the results of these experiments are catalogued. It will be seen that in one of the ten monkeys injected the results were entirely negative (Monkey 135). In two others a tracheobronchitis developed, but the autopsy showed no involvement of the lungs (Nos. 20 and 108). In the remaining seven a *Bacillus influenzæ* pneumonia was definitely established.

Lobes Involved.—The left lower lobe was involved in six cases, and the right lower lobe in six cases. The left upper lobe showed changes

in five of the monkeys, the right upper lobe in only four. All the lobes were affected in two cases (Nos. 124 and 136).

The influenza bacillus was recovered in pure culture from the lungs in two out of the seven cases (Nos. 124 and 138). It was recovered from the trachea or bronchus in three animals (Nos. 124, 136, and 138). It was also cultivated from the trachea in one of the monkeys that failed to develop pneumonia (No. 20). It was isolated from the heart's blood in only one case (No. 138) and that was in the monkey that developed pericarditis in connection with pneumonia.

As will be seen from Table II the lesion in the lungs is described in seven cases as a bronchopneumonia. The solid hepatized lung, so characteristic of pneumococcus infections, was never observed in these animals. The consolidation was distinctly patchy, though the patches were often confluent, especially around the hilum. The hemorrhage and edema frequently seen in *Bacillus influenzæ* pneumonia in man were present, but were usually less intense in the monkey. The picture in some of these cases was doubtless modified to some extent by the comparatively large amount of culture injected into the trachea. This conclusion is supported by the fact that Monkey 155, injected with 4 cc. of killed culture, also developed moderate bronchopneumonic lesions. Apparently the amount of toxic substance present in the culture was capable in itself of setting up a considerable cellular reaction. Monkey 136 received only 1 cc. of peritoneal exudate and developed a markedly hemorrhagic type of pneumonia, similar to that which developed spontaneously in some of the monkeys with experimental influenza.

On removing the sternum in these cases the lungs were invariably found lying free in the pleural cavities. Neither the fibrinous exudate of pneumococcus pneumonia nor the empyema of streptococcus pneumonia was to be seen. On removing the lungs irregular patches of increased resistance could be made out on palpation, most marked around the hilum and along the posterior border where the foci of consolidation usually coalesced to form larger patches. The surface of the lung over these patches was dark red, or even hemorrhagic, and sharply defined from the surrounding pink tissue (Figs. 7 and 8). On section the patches were dark red and rather gelatinous looking. Bloody fluid could be squeezed from the patches of consolidation.

TABLE II.

Monkeys That Received B. influenzæ Intratracheally.

Monkey No.	Date of injection.	Amount injected.	Date killed.	Pathological findings.	Autopsy cultures.
	1919		*1919*		
124	Aug. 11	5 plates (5 cc.).	Aug. 13	Confluent bronchopneumonia, all lobes; tracheobronchitis; hyperplasia of thymus and thoracic lymph glands.	H. B., n. g. R. L., pure B. I. Tr., " " Br., " "
20	" 14	5 (5 ").	" 20	Tracheobronchitis; hyperplasia of thymus and thoracic lymph glands.	H. B., n. g. Tr., pure B. I.
108	" 14	1 plate (1 ").	" 19	Tracheobronchitis; hyperplasia of thymus and thoracic lymph glands; necrosis of peribronchial lymph glands.	H. B., n. g. Tr., "
136	" 14	1 cc. of peritoneal exudate.	" 19	Hemorrhagic bronchopneumonia, all lobes; edema of lungs; tracheobronchitis; bronchiolitis; hyperplasia of thymus, spleen, thoracic and abdominal lymph glands.	H. B., n. g. L. L., " R. L., " Right br., pure B. I.
135	" 14	1 cc. of peritoneal exudate.	Not killed.		
137	" 19	4 plates (4 cc.).	Aug. 21	Confluent bronchopneumonia, R. U., R. L.; tracheobronchitis; hyperplasia of thymus.	H. B., n. g. R. U., " Tr., "

138	Aug. 19	4 plates (4 cc.).	Aug. 21	Confluent bronchopneumonia, R. L., L. U., L. L.; tracheobronchitis; pericarditis; hyperplasia of thymus and spleen.	L. L., pure B. I. Pericard., pure B. I. H. B., pure B. I. Tr., " "
141	" 22	1 plate (1 ").	" 29	Resolving bronchopneumonia, L. U., L. L., R. L.; hyperplasia of thoracic lymph glands; rhinitis; tracheobronchitis.	H. B., n. g. Tr., " Turb., Staph. A.
149	" 23	4 plates (4 ").	" 28	Resolving bronchopneumonia, L. U., L. L., R. U.; hyperplasia of thymus and thoracic lymph glands; bronchiolitis.	H. B., n. g. L. U., " L. L., " Tr., "
152	" 26	1 cc. of peritoneal exudate.	" 27	Bronchopneumonia, R. L., L. L.; tracheobronchitis; hyperplasia of thymus; mediastinitis.	H. B., " R. L., " L. L., " Tr., "
155	" 26	4 plates (4 cc.) of killed *B. influenzæ*.	" 28	Bronchopneumonia, R. L., L. L.; bronchiolitis.	R. L., " Tr., "

H. B., indicates heart's blood.

Areas of compensatory emphysema were frequently observed, especially toward the periphery of the lobe (Fig. 9). The dense new growth of connective tissue, often found in late influenza pneumonia in man, was not present, no doubt for the reason that the monkeys were killed comparatively early in the course of the infection. The trachea and bronchi contained mucopurulent material, in some cases blood-tinged. The tracheal and interbronchial lymph glands were considerably enlarged.

Microscopically, the changes observed were similar to those which have already been described above in connection with spontaneous *Bacillus influenzæ* pneumonia following an attack of experimental influenza. Sections from the lung show disseminated areas of infiltration which are often located about a bronchiole. The lumen of the bronchiole is filled with polymorphonuclear leucocytes and lymphoid cells, and sometimes red blood cells are mixed with the leucocytes (Figs. 10 and 11). The epithelial lining of the bronchioles is partially or perhaps completely destroyed and the underlying tissue is densely infiltrated with leucocytes (Fig. 11). This infiltration extends out into the adjacent interstitial tissue and the contiguous alveolar walls (Fig. 12) which show intense engorgement of the capillaries. The alveoli in this neighborhood do not escape, but contain red blood cells, leucocytes, and desquamated epithelial cells. At times the engorgement of the alveolar walls is so severe that large numbers of red blood cells extravasate into the alveolar lumen. These foci of exudation may coalesce to form larger areas, but the process never reaches the stage of universal consolidation as seen in pneumococcus infections. Vesicular emphysema is present, but is usually not so striking as that in man. The larger bronchi contain pus and the columnar epithelial cells take on the goblet form. Pus cells are found working their way out between the epitheiial cells lining the bronchus, and the submucosa is infiltrated with polymorphonuclear leucocytes and lymphoid cells. The pleura shows little if any deposit of fibrin. There is a striking absence of perivascular infiltration of leucocytes and of the lymphatic involvement, which were a prominent feature in pneumococcus and streptococcus pneumonia.

The following case is an excellent example of the findings after experimental *Bacillus influenzæ* pneumonia.

Protocol 5.—Monkey 136. *Cebus capucinus*, male; weight 1,100 gm. Aug. 14, 1919. Received intratracheally 1 cc. of exudate from peritoneum of monkey with *B. influenzæ* peritonitis. Aug. 19. Killed by blow on head.

Anatomical Diagnosis.—Experimental *B. influenzæ* bronchopneumonia; hemorrhagic edema of lungs; acute tracheobronchitis; bronchiolitis; hyperplasia of the thymus gland; hyperplasia of spleen, thoracic, mesenteric, and retroperitoneal lymph glands.

Autopsy.—Performed Aug. 19. On removing the sternum the lungs are found lying free in the pleural cavities, which are free from adhesions and fluid. The pericardial sac is normal. The anterior surface of the pericardium is partially covered by a greatly enlarged thymus gland, which measures about 0.5 cm. in thickness (Fig. 8). The gland is somewhat triangular in shape with the apex of the triangle pointing upward and the base of the triangle lying transversely across the pericardium. Cut surface of the gland is pale pinkish yellow and homogeneous, with definite lobules. Heart not enlarged. Myocardium and valves normal. Right lung is voluminous. Surface of all three lobes is smooth and glistening and everywhere mottled with small bright red hemorrhagic areas lying close together, in some places confluent, in others separated by narrow gray zones which apparently mark the boundaries between the lobules (Figs. 7 and 8). This hemorrhagic condition is also marked at the base and over the anterior surface of the lobes. The lobes have a boggy consistence and contain considerably less air than a normal lung, but the tissue has not the firm resilient consistence offered by a pneumococcus consolidation. Cut surface of right lower lobe shows engorgement and marked hemorrhagic edema. In the upper and middle lobes the congestion is noticed at the root of the lung and gradually fades out toward the periphery. The upper and middle lobes are similar to the lower lobe but show less involvement. The left lung is similar to the right in respect to its surface. The lower lobe shows the same striking hemorrhagic appearance, and the upper and middle lobes are involved to a less extent. Cut surface of the left lung is similar in general to that of the right. Tracheal and interbronchial lymph glands are enlarged and intensely congested. Trachea and bronchi are filled with bloody frothy mucus. Spleen is definitely enlarged. Cut surface is dark red. Liver and kidneys show cloudy swelling. Suprarenals normal. The retroperitoneal and mesenteric lymph glands are considerably enlarged. Pancreas shows no noticeable changes.

Cultures.—Right lower lobe, no growth; left lower lobe, no growth; right bronchus, pure culture of *B. influenzæ*.

Microscopic Examination.—*Right upper lobe.*—Intense engorgement of all blood vessels and capillaries. In many places the alveolar walls are greatly thickened on account of the distention of capillaries with red blood cells. In lower half of section there is a small patch of consolidation where the alveoli and their walls are infiltrated with many polymorphonuclear leucocytes. In addition to leucocytes the alveoli contain desquamated epithelial cells and red blood corpuscles. The bronchioles contain pus, and the walls of the bronchioles are infiltrated with leucocytes. Section stained for bacteria shows no influenza

bacilli or other bacteria. *Left lower lobe.*—Section shows engorgement, hemorrhage, and, in some places, considerable emphysema (Fig. 9). Considerable number of red blood corpuscles found in the alveoli. Stained section shows no bacteria. No leucocytic exudate in the alveoli in this section. *Left middle lobe* (*Fig. 10*).—Shows marked engorgement and hemorrhage similar to that in the left lower lobe. *Thymus.*—The follicles are greatly enlarged, due to hyperplasia of the chyme centers and to the greatly increased number of lymphoid cells. Sinuses are distended, being packed with lymphoid cells. The fatty tissue between the follicles is compressed to a thin layer. No polymorphonuclear leucocytes or bacteria are seen. *Trachea.*—Shows no striking changes. The capillaries beneath the epithelium are engorged. The epithelial cells are swollen and filled with mucus. There is a thin layer of mucus on the epithelial surface in which a few red blood corpuscles and lymphocytes are seen. There is no hemorrhage or cellular infiltration in the submucosa. *Tracheal lymph node.*—Greatly enlarged. The vessels and capillaries are intensely engorged. The follicles show hyperplasia. The lymph channels are distended and filled with lymphoid cells and red blood corpuscles. Many of them contain coagulated plasma. In places there is considerable hemorrhage into the follicles. *Kidneys.*—Appear normal, except for moderate capillary engorgement. *Suprarenals.*—Normal. *Spleen.*—There is a very marked lymphoid hyperplasia, the follicles being greatly swollen, very prominent, and densely packed with lymphoid cells. There is slight congestion of the blood vessels and capillaries. The sinuses are filled with lymphoid cells.

Bacillus influenzæ Pericarditis.—One of the monkeys with experimental *Bacillus influenzæ* pneumonia developed an acute pericarditis (No. 138). Cultures from the pericardial fluid gave a pure growth of *Bacillus influenzæ.* The case is of such unusual interest that the autopsy findings will be given in detail.

Protocol 6.—Monkey 138. *Macacus syrichtus*, female; weight 2,915 gm. Aug. 19, 1919. Injected intratracheally with 4 cc. (four chocolate agar plates) of *B. influenzæ.* Aug. 21. Killed by blow on head.

Anatomical Diagnosis.—Experimental bronchopneumonia (*B. influenzæ*), left upper and lower and right lower lobes; acute serofibrinous pericarditis; chronic fibrous pleuritis, right side; acute tracheobronchitis; hyperplasia of thymus and spleen; focal necrosis in thymus; cloudy swelling of viscera.

Autopsy.—Performed Aug. 21. Subject is a medium sized female *Macacus.* On removing the sternum thymus gland is found considerably enlarged. There are a few adhesions in the right pleural cavity. Left pleural cavity normal. On opening the pericardium a moderate quantity of cloudy fluid escapes. Both the visceral and parietal layers of the pericardium are opaque and show marked edema, but there is very little fibrin. Heart moderately enlarged. Myocardium and valves normal. Left lung lies free in the pleural cavity. Anterior portions appear nor-

mal. Posteriorly, however, there are grayish red translucent patches of consolidation. These areas involve a considerable part of the lower lobe. In the upper lobe the process is less extensive. In both cases the changes appear to start at the hilum and spread outward. The right lung is bound to the chest wall by old fibrous adhesions. On section upper and middle lobes are normal. Lower lobe shows a small patch of pneumonia, similar to that found in the left lung. Tracheal and bronchial mucous membrane is congested and covered with mucus. Liver and kidneys show cloudy swelling. Other abdominal organs appear normal.

Cultures.—Left lower lobe, pure culture of *B. influenzæ*; pericardium, pure culture of *B. influenzæ*; heart's blood, pure culture of *B. influenzæ*; trachea, pure culture of *B. influenzæ*.

Microscopic Examination.—*Trachea.*—Mucus on epithelial surface. Moderate number of lymphoid cells and polymorphonuclear leucocytes in submucosa. Leucocytes also found between epithelial cells. Another section at bifurcation of trachea shows mucus and pus cells on the mucosa and infiltration similar to that in the other section. *Right lower lobe.*—Sections show extensive infiltration of leucocytes in the peribronchial tissue and in the adjacent alveoli. The exudate consists of polymorphonuclear leucocytes, lymphoid cells, and, in addition to these, the affected alveoli contain desquamated epithelium, and a slight amount of fibrin in some places. Bronchi and bronchioles filled with pus. Deposit of fibrin and leucocytes on the pleura. Perivascular infiltration is not observed. Section stained for bacteria shows many minute Gram-negative bacilli (*B. influenzæ*) in the alveolar exudate, mostly intracellular, being in the leucocytes. No influenza bacilli are found in the alveolar walls. Bronchioles are filled with pus, with considerable destruction of epithelium. Another section from right lower lobe shows interstitial pneumonia. *Left lower lobe.*—Shows infiltration similar to that on right side; small plugs of fibrin in some of the alveoli. Another section shows numerous alveoli filled with dense plugs of fibrin, in which is suspended a moderate number of disintegrating leucocytes. A moderate number of intracellular influenza bacilli is found. This lobe shows bronchopneumonia. A few influenza bacilli are found in the alveoli. *Thymus.*—Marked hyperplasia of the follicles and of the chyme centers. The lymph channels are greatly distended and are filled with coagulated plasma. The connective and adipose tissue surrounding the gland is infiltrated at many points with polymorphonuclear leucocytes. There is an area of necrosis of considerable extent at one point in the parenchyma. *Pericardium and heart muscle.*—Serosa of the pericardium is covered with a thick vascular membrane of granulation tissue on the surface of which there is a deposit of fresh fibrin and polymorphonuclear leucocytes. At some points the exudate is necrotic. There is moderate infiltration of leucocytes in the superficial portion of the myocardium. The muscle fibrillæ show cloudy swelling. *Liver.*—Hydropic degeneration about periphery of lobules. *Kidney.*—Shows definite cloudy swelling; glomeruli congested. *Spleen.*—Hyperplasia of follicles.

Other Pathological Changes Found in Association with Experimental Bacillus influenzæ Pneumonia.—Tracheobronchitis was noted in five out of the seven cases. It differed in no way from the tracheobronchitis already described in connection with experimental influenza. Hyperplasia of the thymus was observed in six of the seven cases, and was also present in the two monkeys (Nos. 20 and 108) which developed only tracheobronchitis following the intratracheal injection of influenza bacilli. The microscopic changes were identical with those already described under experimental influenza.

Enlargement of the tracheal and interbronchial lymph glands was usually noted, and in one case (No. 136) the various groups of abdominal lymph glands also shared in this hyperplasia. Monkey 108 showed focal necrosis in the bronchial lymph nodes, and in Monkey 152 there was an acute mediastinitis. There was hyperplasia of the spleen in two instances (Nos. 136 and 138).

Cloudy swelling of the liver was quite common, and similar changes, though usually less marked, were observed in the kidneys, adrenals, and heart muscle. The testicle showed no degenerative changes. No foci of necrosis were found in the rectus muscles.

Bacillus influenzæ Pneumonia in Man.

Microscopic sections from a number of cases of pure *Bacillus influenzæ* pneumonia in man[6] have been studied in connection with experimental *Bacillus influenzæ* pneumonia in monkeys. Photomicrographs from the lungs of two of the cases are shown in Figs. 13 and 14. A number of the lesions described by Wolbach are well displayed in these reproductions.

The first section (Fig. 13) is from a patient who died on the 13th day of the disease. At autopsy the lungs showed confluent bilateral bronchopneumonia and bronchiectasis. Microscopically, as the photograph shows, there are the characteristic changes in the bronchioles described above. The lumen is plugged with an exudate of leucocytes and red blood cells and the lining epithelium has been destroyed in part. The wall of the bronchiole and the adjacent structures are engorged with red blood cells and infiltrated with leucocytes.

[6] These sections were obtained through the kindness of Dr. S. B. Wolbach.

The alveoli contiguous to the bronchiole are filled with coagulated plasma in which are imbedded leucocytes, red blood cells, and desquamated epithelium. Alveoli further distant from the bronchiole contain coagulated plasma but are mostly free from exudate. The alveolar walls are everywhere engorged with red blood cells.

The second patient died of *Bacillus influenzæ* pneumonia on the 9th day of the disease. The autopsy showed confluent bronchopneumonia of both lungs and hemorrhagic myositis of the pectoral and rectus muscles. Microscopic sections of this case (Fig. 14) show severer lesions than those of the previous case. Engorgement, hemorrhage, and edema are present, but in addition there is an extensive emphysema. A moderate grade of leucocytic infiltration is also noted, many of the leucocytes being of the small mononuclear type. The peculiar hyaline membrane of fibrin described by Wolbach is found lining many of the distended alveoli.

The lesions found in these two cases of *Bacillus influenzæ* pneumonia in man are closely simulated by those observed in the lungs of Monkey 157, which, it will be recalled, developed an attack of *Bacillus influenzæ* pneumonia following experimental influenza.

DISCUSSION.

The pathological changes observed in experimental influenza in monkeys differ in no essential respect from those which occur in human influenza. In both instances the disease manifests itself as an acute catarrhal inflammation of the upper respiratory tract, extending usually from the nasal cavities down into the trachea and bronchi. In both diseases the accessory sinuses are often involved and a secondary bronchopneumonia is a frequent complication.

If, however, experimental influenza in monkeys resembles human influenza, the analogy between experimental *Bacillus influenzæ* pneumonia in monkeys and spontaneous *Bacillus influenzæ* pneumonia in man may be said to be still more striking. For in the case of *Bacillus influenzæ* pneumonia in man there are certain characteristics which differentiate it from the pneumonia excited by other bacteria, and render it readily recognizable by the pathologist. The studies of Pfeiffer, and more recently those of MacCallum and Wolbach, have

defined these peculiar lesions very clearly. The intense engorgement, hemorrhage, and edema, the purulent bronchiolitis, the scattered foci of peribronchiolar consolidation, and the ensuing emphysema and bronchiectasis have been emphasized by these writers as anatomical marks which would distinguish the infection from ordinary lobar and lobular pneumonia of pneumococcus and streptococcus origin.

In the foregoing experiments it has been shown that by the injection into monkeys of virulent influenza bacilli, a type of pneumonia can be induced that corresponds in most respects with that which results from *Bacillus influenzæ* infection of the lungs in man. There are the same characteristic changes in and about the bronchioles, the same hemorrhage and edema, the same focal consolidation, and the same tendency toward emphysema and bronchiectasis in the later stages.

Experimental *Bacillus influenzæ* pneumonia in monkeys differs in one respect from the spontaneous disease in man. The process in monkeys appears to be the expression of a milder infection than that in man. The engorgement and edema are usually less marked in monkeys and the leucocytic infiltration is more pronounced. The difference, however, might reasonably have been expected. The human cases occurred during a very severe epidemic in which *Bacillus influenzæ* had presumably acquired exceptional virulence. The strain which was employed for producing the disease experimentally had been cultivated for weeks on artificial media and it was only with considerable difficulty that even a moderate grade of virulence was conferred on it for monkeys. Moreover, in some of the animals the experimental lesions were induced by massive doses of bacteria which might have possessed enough toxicity to excite independently a certain amount of cellular reaction. That massive doses alone are capable of setting up considerable inflammatory reaction is evidenced by Monkey 155, which received 4 cc. of killed influenza bacilli intratracheally and developed a mild bronchopneumonia with some exudation into the alveoli. In this connection it is interesting to note that when *Bacillus influenzæ* pneumonia developed spontaneously in monkeys as a complication of experimental influenza, the pathological changes of *Bacillus influenzæ* pneumonia in man were even more closely simulated than in the animals in which the disease was produced experimentally by direct intratracheal injection of massive

amounts of culture. Monkey 157, for example, developed *Bacillus influenzæ* pneumonia subsequent to the inoculation of *Bacillus influenzæ* in the nose and throat. The autopsy showed in addition to the peribronchial changes, hemorrhage, edema, emphysema, and bronchiectasis. The most characteristic changes were also observed in the lungs of the monkeys that were inoculated intratracheally with small amounts of culture.

The pathogenesis of *Bacillus influenzæ* pneumonia is apparently different from that of pneumococcus and streptococcus pneumonia. In both the experimental and the spontaneous disease the infection seems to travel down the bronchial tree into the bronchioles and subsequently involves the neighboring alveoli by contiguity. In pneumococcus and streptococcus pneumonia the bacteria penetrate the bronchial mucous membrane almost at once and spread rapidly through the perivascular lymph spaces to the alveolar walls in all parts of the lobe. In *Bacillus influenzæ* pneumonia the bacteria are present in large numbers in the bronchioles and to a less extent in the adjacent alveoli. Influenza bacilli, however, are rarely found in any part of the interstitial tissue, and, as MacCallum has pointed out, the lymphatics are noticeably free from involvement in *Bacillus influenzæ* pneumonia. This may explain why the influenza bacillus rarely invades the blood stream. The influenza bacillus lacks the invasive character possessed by the pneumococcus and streptococcus; consequently the lesions produced by the first organism are focal, while those produced by the last two usually involve extensive areas. The influenza bacillus, growing, in a sense, outside the body, injures chiefly contiguous parts, probably by means of toxic substances which it secretes; the pneumococcus and streptococcus, invading the lymphatics, reach all parts of the lung and excite a massive lesion. MacCallum believes that suppurative bronchiolitis, with peribronchial pneumonia, may be caused in some instances by the streptococcus. There is always a possibility, however, that in such cases the influenza bacillus has been the first to infect the lung and has later been supplanted by the streptococcus as a secondary invader. In view of the experiments reported in this paper such an explanation seems fairly plausible. In a number of cases of experimental *Bacillus influenzæ* pneumonia sterile cultures were obtained from the lungs at autopsy,

but there was no question as to the etiology of the pneumonic process. The influenza bacillus had produced its characteristic inflammatory reaction and had then been overcome by the resistance of the animal.

CONCLUSIONS.

1. Virulent influenza bacilli, when injected into the nose and throat of monkeys (*Cebus capucinus* and *Macacus syrichtus*), excite an acute inflammation of the upper respiratory tract, characterized by swelling and hyperemia of the mucous membrane, infiltration of the mucosa and submucosa with leucocytes, desquamation of epithelial cells, and the production of a mucopurulent exudate. The accessory sinuses are often implicated in the infection.

2. Experimental *Bacillus influenzæ* infections of the upper respiratory tract are frequently accompanied or followed by bronchiolitis, peribronchial infiltration, and bronchopneumonia with hemorrhage and edema in the early stage, emphysema and bronchiectasis in the later stages. In general, the process closely resembles uncomplicated *Bacillus influenzæ* pneumonia in man.

3. The injection of virulent influenza bacilli directly into the trachea of monkeys induces in them an experimental bronchiolitis and hemorrhagic bronchopneumonia, similar in all respects to spontaneous *Bacillus influenzæ* pneumonia.

4. In experimental *Bacillus influenzæ* infections of either the upper or lower respiratory tract the influenza bacillus can usually be recovered during the acute stage by culture, either pure or in association with other bacteria.

5. In experimental *Bacillus influenzæ* infections in monkeys characteristic changes occur in the thymus gland—hyperplasia of the follicles, distention of the lymphatic channels, and infiltration of the parenchyma with leucocytes. This enlargement appears to be merely part of a general hyperplasia of the lymphoid structures in the cervical and thoracic regions.

EXPLANATION OF PLATES.

PLATE 90.

FIG. 1. Monkey 139. Experimental influenza. Section of a turbinate showing engorgement of capillaries in the submucosa, and profuse exudate of mucus and pus cells on the surface of the epithelium. × 100.

FIG. 2. Monkey 159. Experimental influenza followed by *B. influenzæ* pneumonia. Section from the trachea showing infiltration of leucocytes in the mucosa and submucosa. Deposit of mucus and leucocytes on the surface. × 400.

PLATE 91.

FIG. 3. Monkey 137. Experimental *B. influenzæ* pneumonia. Section of the trachea showing acute tracheitis. × 400.

FIG. 4. Monkey 157. Experimental influenza followed by *B. influenzæ* pneumonia. Section from the right upper lobe showing hemorrhage, engorgement, and emphysema. The alveoli contain fibrin and many red blood cells, also a few leucocytes and desquamated epithelial cells. × 100.

PLATE 92.

FIG. 5. Monkey 157. Experimental influenza followed by *B. influenzæ* pneumonia. Section from the right upper lobe showing bronchiolitis, peribronchial infiltration of leucocytes, hemorrhage, and edema. *Cf.* with Fig. 13. × 100.

FIG. 6. Monkey 157. Experimental influenza followed by *B. influenzæ* pneumonia. Section through the entire upper right lobe, showing peribronchial pneumonia, bronchiolitis, marked bronchiectasis, and emphysema. × 3.

PLATE 93.

FIG. 7. Monkey 136. Experimental *B. influenzæ* pneumonia. Hemorrhagic and edematous infiltration. This photograph illustrates the patchy character of the consolidation.

FIG. 8. Monkey 136. Anterior view showing hyperplasia of the thymus, which extends down over the greater part of the pericardial sac.

PLATE 94.

FIG. 9. Monkey 136. Experimental *B. influenzæ* pneumonia. Section through the entire left lower lobe showing hemorrhage, engorgement, and edema, with emphysema. × 3.

FIG. 10. Monkey 136. Experimental *B. influenzæ* pneumonia. Left middle lobe. Bronchiolitis, peribronchiolitis, engorgement, and hemorrhage. × 100.

PLATE 95.

FIG. 11. Monkey 124. Experimental *B. influenzæ* pneumonia. Section of the left lower lobe showing purulent bronchiolitis, partial destruction of the wall of a bronchiole, and peribronchiolar pneumonia. × 130.

PLATE 96.

FIG. 12. Monkey 152. Experimental *B. influenzæ* pneumonia. Section from the left lower lobe showing infiltration of leucocytes in the alveoli and septa adjacent to terminal bronchioles. × 100.

FIG. 13. *B. influenzæ* pneumonia in man (Dr. Wolbach's case). Acute bronchiolitis and peribronchiolar infiltration of leucocytes; engorgement and edema. *Cf.* with Fig. 5. × 100.

PLATE 97.

FIG. 14. *B. influenzæ* pneumonia in man (Dr. Wolbach's case). Extensive hemorrhage, engorgement, and edema; hyaline membrane lining alveoli; emphysema. × 130.

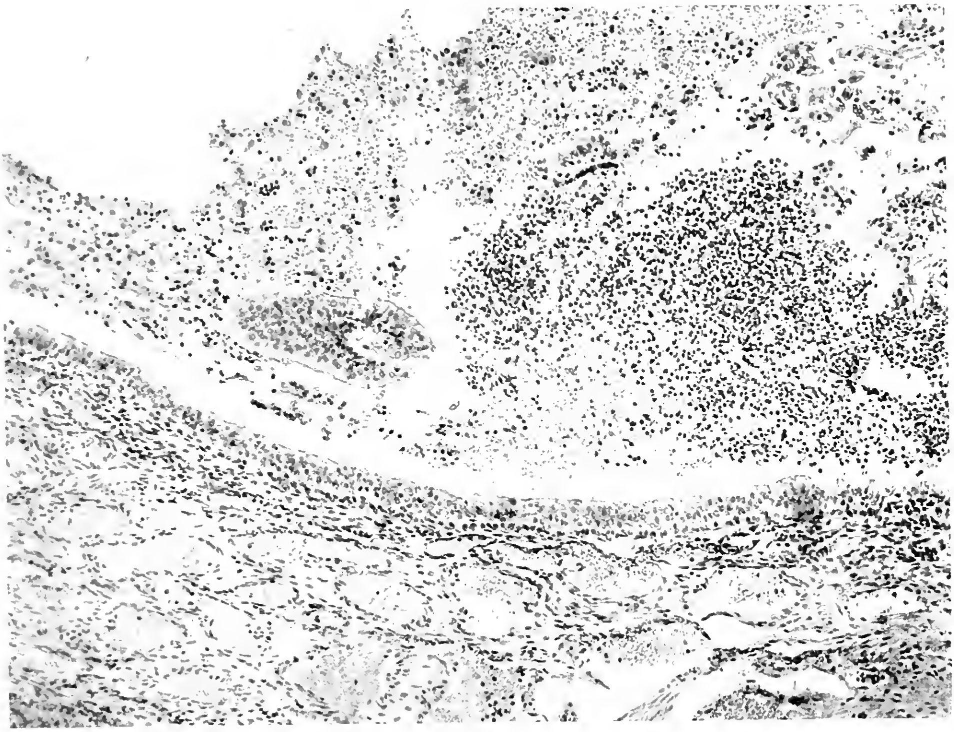


Fig. 1.

Fig. 2.

(Cecil and Blake: Experimental pneumonia. X.)

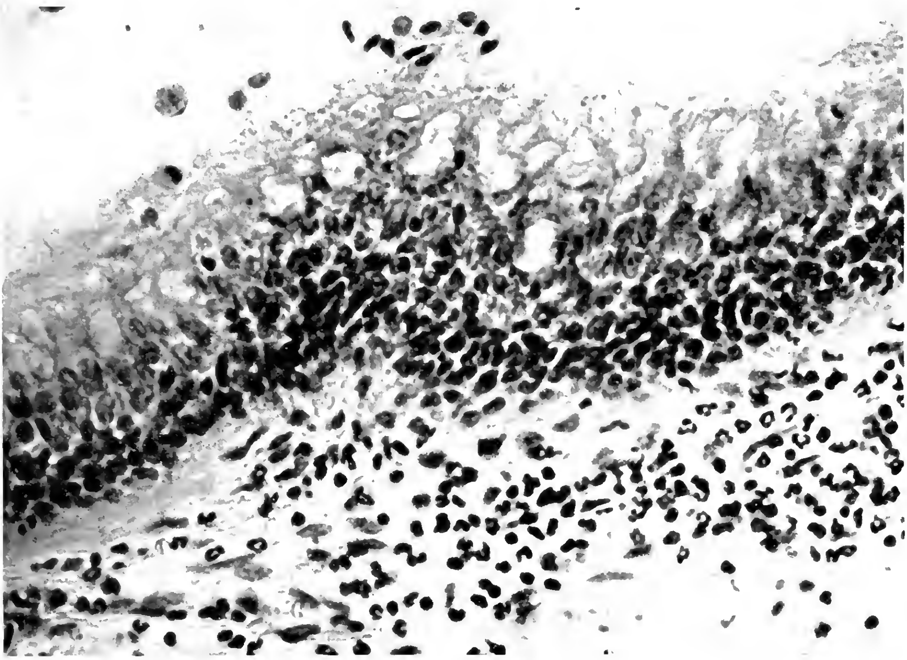


FIG. 3.

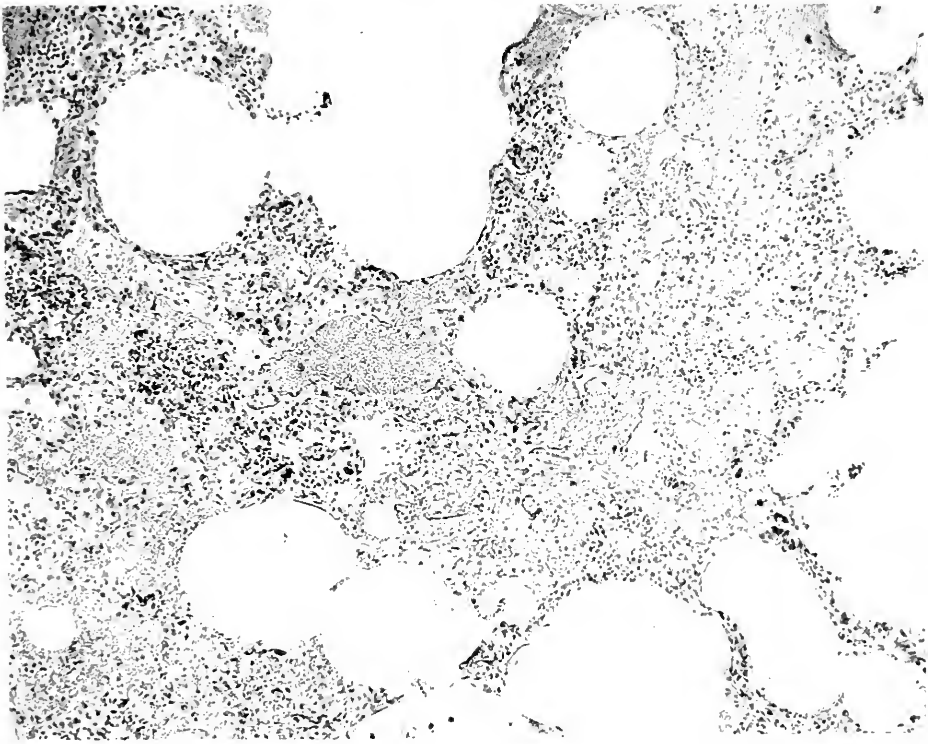


FIG. 4.

(Cecil and Blake: Experimental pneumonia. X.)

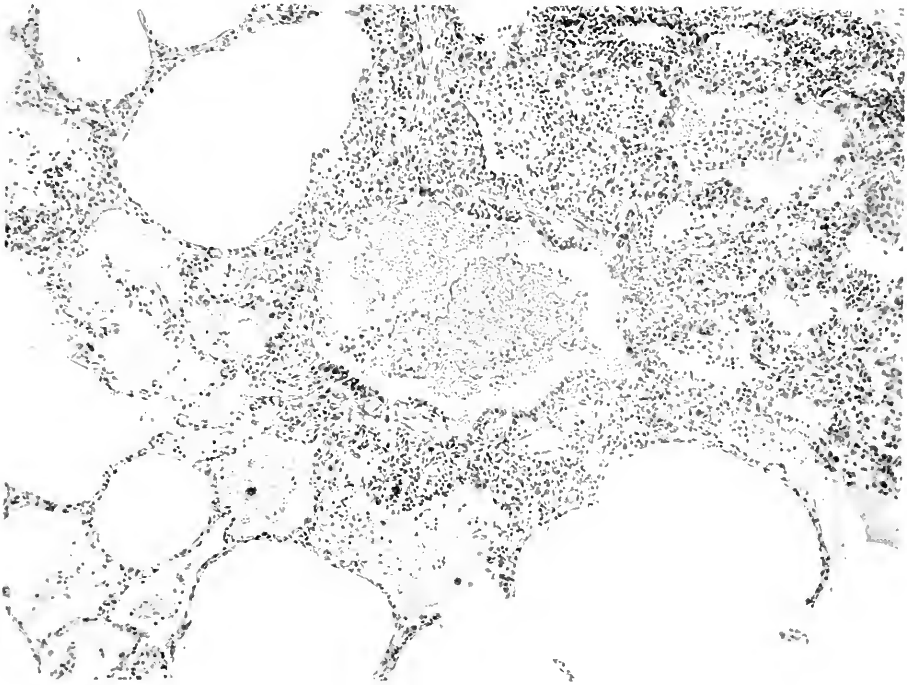


FIG. 5.

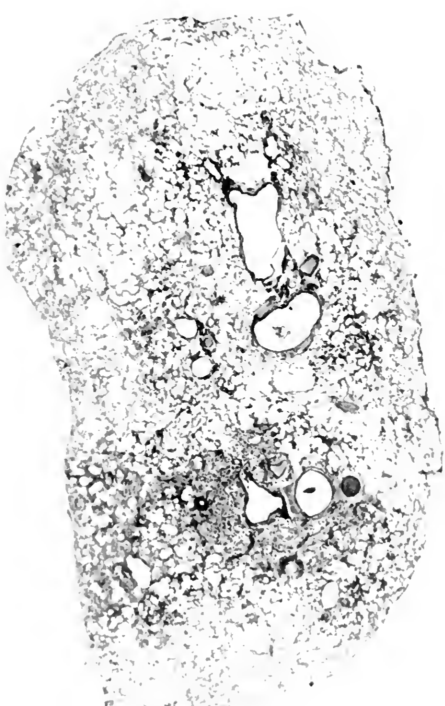


FIG. 6.

(Cecil and Blake: Experimental pneumonia. X.)

FIG. 8.

FIG. 7.

(Cecil and Blake: Experimental pneumonia. X.)

FIG. 9.

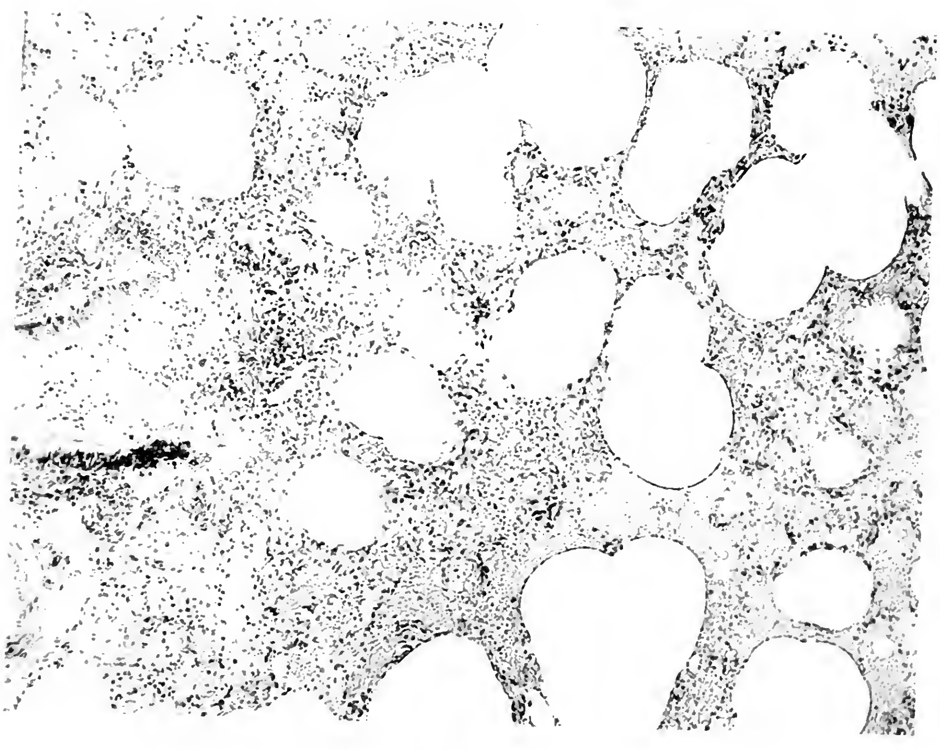


FIG. 10.

(Cecil and Blake: Experimental pneumonia. X.)

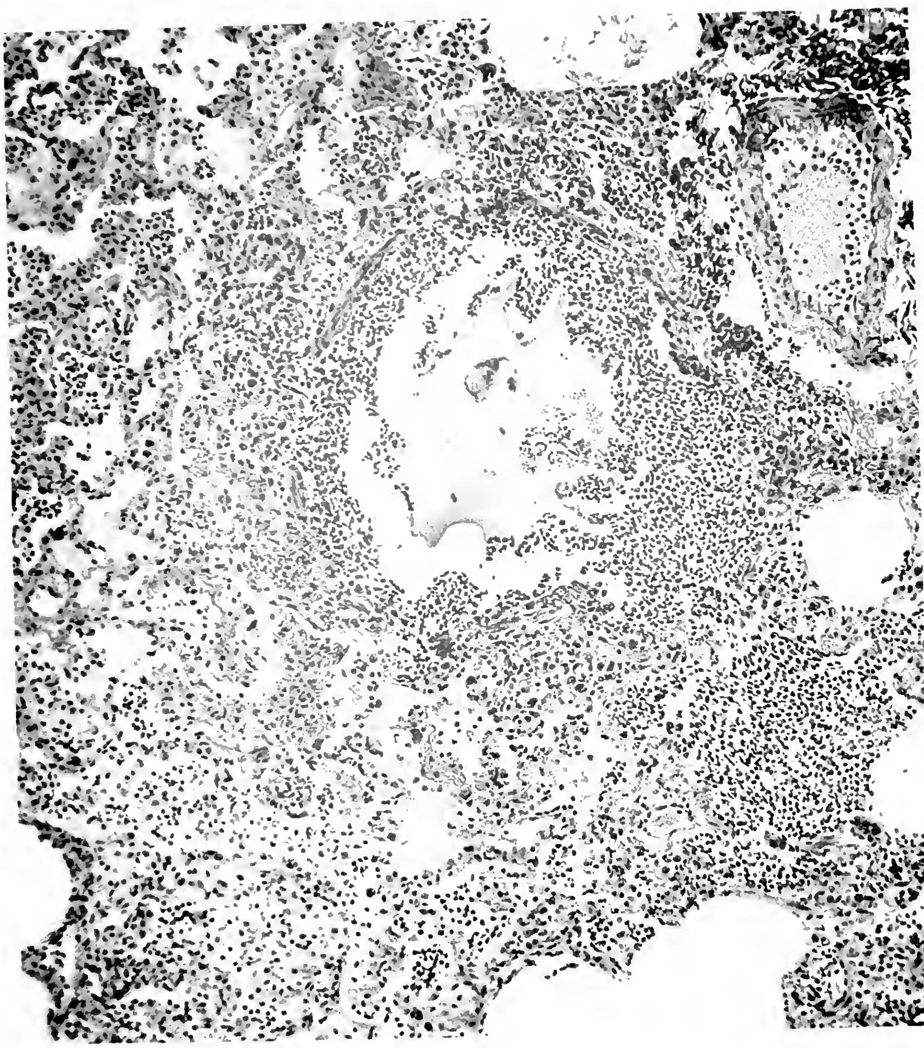


FIG. 11.

(Cecil and Blake: Experimental pneumonia. X.)

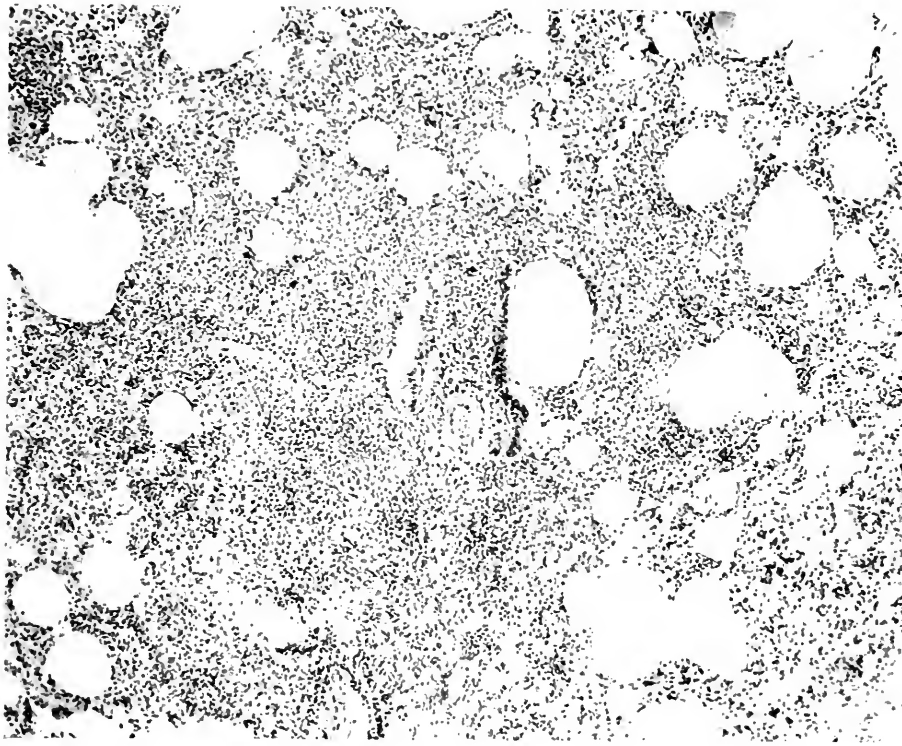


Fig. 12.

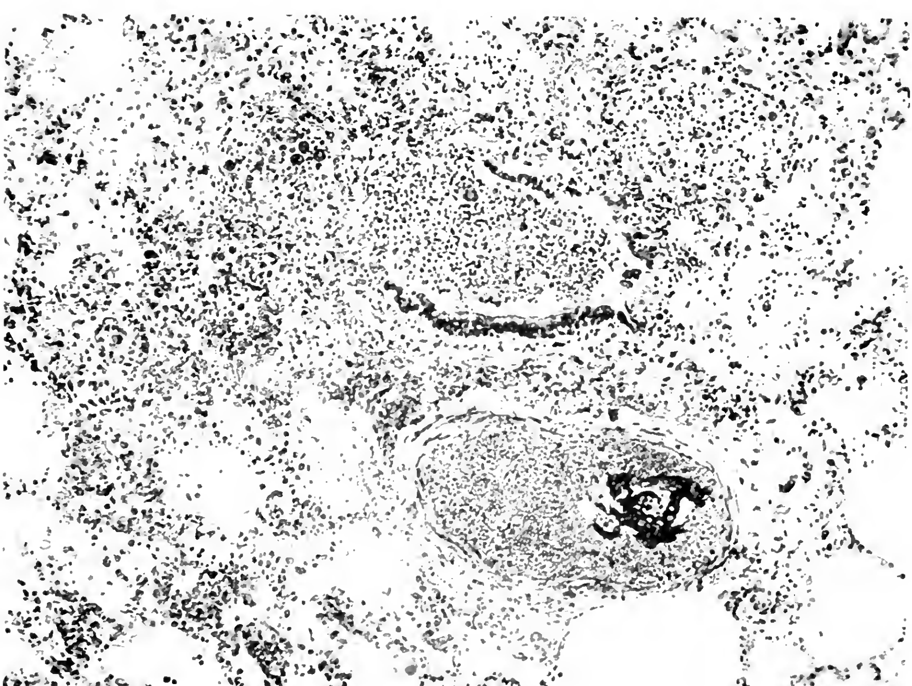


Fig. 13.

(Cecil and Blake: Experimental pneumonia. X.)

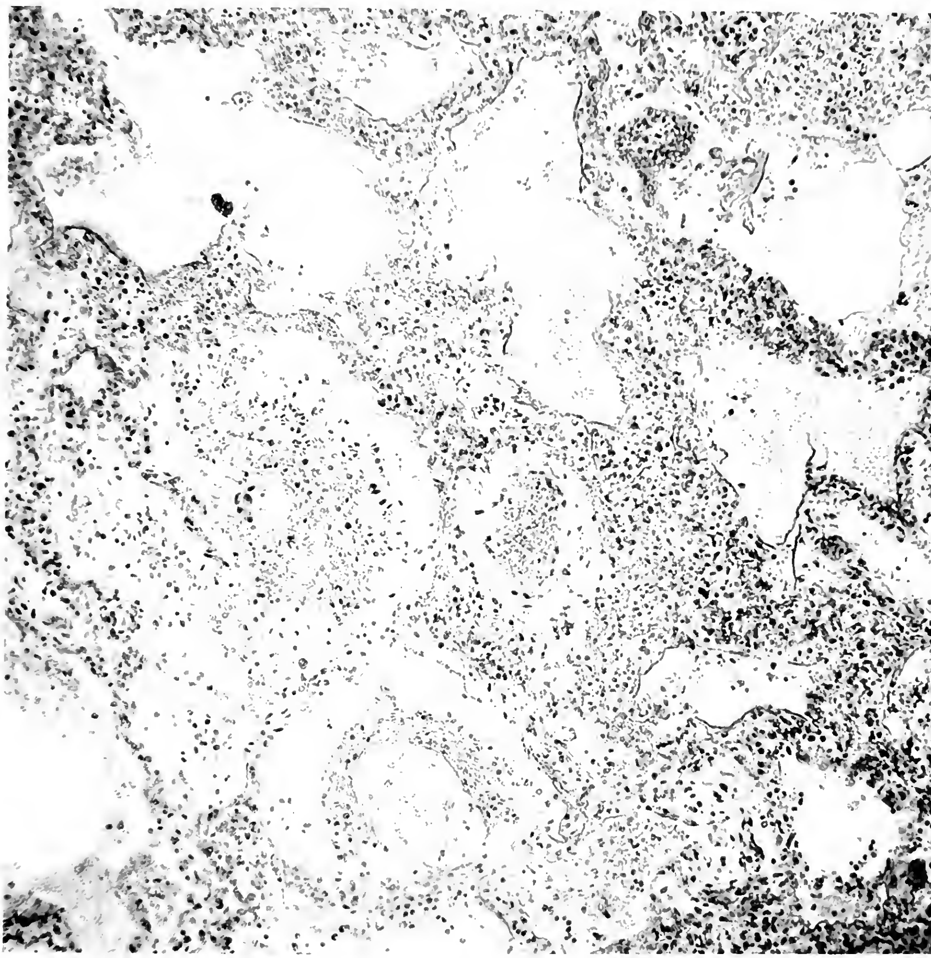


FIG. 14.

(Cecil and Blake: Experimental pneumonia. X.)

EXPERIMENTAL STUDIES ON HETEROPLASTIC BONE FORMATION.*

By GOICHI ASAMI, M.D., and WILLIAM DOCK.

(From the Department of Pathology of Washington University School of Medicine, St. Louis.)

Plates 98 to 100.

(Received for publication, June 22, 1920.)

The occurrence of bone in abnormal locations has been frequently reported in the literature. Its experimental production in various species of animals has also been recorded by a number of workers. Recently one of us (1) described a case of bone formation in the Fallopian tube and gave a discussion of the possible mode of its origin, based upon a review of the literature and a clinical and histological study of the case. At that time it was observed that most of the experimental work done was in connection with different problems, such as the origin of the blood cells and the restoration of renal function, and that for this reason reliable and convincing data on the mode of origin and growth of aberrant bone were not available. Though almost all investigators agreed that such a bone arises from fixed tissue cells by a process of metaplasia, there were great differences of opinion as to the factors initiating the process, and as to whether the proliferating cells become osteoblasts or merely a hyaline connective tissue which later is directly converted into osseous tissue.

The view credited to von Hansemann (2) that aberrant bone formation owes its origin to misplaced islands of perichondrium or cartilage was early rejected as insufficient to explain the frequent occurrence of bone in sclerotic vessels, in necrotic and calcified areas in the lungs, the gastric mucosa, and in other organs in no way associated in their development with cartilage. From a study of pathological specimens Pollack (3), Mönckeberg (4), Bunting (5), Buerger and Oppenheimer (6), and others (7) presented what they believed to be histological evidences of metaplasia. By experimental investigation Sacerdotti and Frattin

* Presented in abstract at the 69th meeting of the Washington University Medical Society, St. Louis, May 3, 1920.

(8), Liek (9), and Poscharissky (10) showed that bone can be produced in the rabbit kidney by ligating the vessels. Maximow (11) described various stages in this bone formation in the rabbit kidney in connection with his studies on the histogenesis of blood cells. Pearce (12) described similar bone formation in the dog kidney after removal of bits of its tissue, and concluded that the bone was produced by the direct ossification of scar tissue. In the aorta of the rabbit Harvey (13) was able to produce bone as well as cartilage by painting the external wall with a solution of silver nitrate in some cases and with one of cupric sulfate in others.

In every instance in which bone was produced in abnormal locations, there has been observed a proliferation of connective tissue cells and capillaries which seemed to migrate into the areas of necrosis and calcification. Bunting and others have favored the theory that the calcium salts act as a stimulus which induces the young connective tissue cells to differentiate into bone-forming cells. While the majority of writers on this subject thus believed the connective tissue cells to be chiefly concerned in bone formation, Thoma (14) was of the opinion that the osteogenic cells are derived from the endothelial cells of the capillaries that erode the calcific placques.

The rather frequent occurrence of cartilage in cases of heteroplastic bone formation as well as in Harvey's experiment suggested that possibly a primary conversion of connective tissue cells into cartilage may take place in some instances with subsequent formation of osseous tissue. Such a possibility was indicated also in the case of the Fallopian tube referred to above.

The bone marrow in aberrant bone was believed by Marchand (15) to arise from marrow cells transported through the blood stream, while Maximow was of the opinion that it was derived from the wandering cells or large lymphocytes, and Bunting thought it to be the result of a metaplasia of connective tissue cells.

It is thus apparent that although numerous observations both on human pathological specimens and animal experiments have been published, the opinions based upon them are conflicting. Owing to the accidental nature of the experimental investigations thus far carried out, detailed descriptions of the processes involved in the aberrant bone formation are not available. We have, therefore, decided to carry out further investigations as regards the mode of origin, growth, and detailed structures of the bone, as well as the probable part played by cartilage and calcium salts in initiating this bone formation.

Material and Methods.

Thirty-nine rabbits were divided into three series. In the first series of twenty animals renal vessels were ligated on one side.[1] In all but one animal the left side was chosen. In two of the animals the ureter was included in ligation and resection. In the second series of fourteen rabbits a portion of the ear and xiphoid cartilages was resected and transplanted into the subcutaneous tissue of the same animal. In a few instances the cartilages transplanted were previously boiled in water. The five animals comprising the third series were subjected to subcutaneous and intramuscular injections of calcium salts, consisting of calcium chloride and calcium carbonate, and of sodium phosphate.

All the animals were killed at various intervals in order to follow progressively the changes that were brought about by the experiments. The tissues were fixed in 4 per cent formaldehyde solution, decalcified in a mixture of phloroglucin and nitric acid, embedded in celloidin, and stained in hematoxylin and eosin. In some instances the Weigert-Van Gieson method of staining was resorted to in order to differentiate the tissues. Many of the kidneys were sectioned *in toto* for the purpose of studying different areas of the kidneys.

Series 1. Ligation of Renal Vessels.

Experimental Data.

Animal 1. 3 Days after Ligation.—The left kidney appears to be practically normal in size. It is dark red in color and soft in consistency and cuts easily throughout its entire extent.

Microscopic Examination.—The parenchyma of the kidney appears definitely hyaline with nuclei more pycnotic than normally. Hyaline casts occupy the lumina of the tubules. The capsule is edematous and infiltrated with lymphocytes, and presents a beginning fibroblastic proliferation. The transitional epithelium of the calices is desquamated *en bloc* at various places, and the cells are somewhat hyaline. Granular calcium deposits are noted along a layer of leucocytes which occur close under the capsule.

Animal 2. 7 Days after Ligation.—The left kidney is somewhat larger than the right and is very soft. The capsule is thickened and the cortex pale. There is a gritty sensation on cutting through the organ.

---

[1] All operations were performed under ether or urethane anesthesia.

Microscopic Examination.—Appearance similar to that in the previous animal except that the hyaline degeneration of the renal tissue is more pronounced and the epithelium of the calices appears healthy at places. Calcium deposits are noted under the capsule.

Animal 3. 14 Days after Ligation.—The left kidney is smaller than the right, soft to touch, and grayish in color. A slight gritty sensation is elicited upon cutting through the tissues.

Microscopic Examination.—Sections show an invasion of the hyaline cortex by fibroblasts from the capsule, accompanied by lymphocytes and capillary blood vessels. The granular calcium deposits have increased slightly in amount and are confined chiefly to the outer borders of the cortex.

Animal 4. 27 Days after Ligation.—The left kidney is markedly diminished in size and is completely surrounded by a mass of fatty tissue. The cortex is hard and cuts with difficulty. The ureter is grayish white and elastic. The pelvis of the kidney contains a soft, elastic, grayish white tissue mass.

Microscopic Examination.—An increased amount of calcium deposits is noted in the cortex and also a slight increase in the medulla. The cortex is invaded by a fresh granulation tissue with numerous lymphocytes.

Animal 5. 28 Days after Ligation.—The left kidney is slightly smaller than the right and appears grayish red. The capsule is surrounded by a mass of fat. The organ cuts with a distinctly gritty sensation. The region about the pelvis is filled with a grayish white, pliable mass of tissue. No bone can be made out on gross inspection.

Microscopic Examination.—Sections show calcific deposits in the cortex and in the medulla close to the calices. No noticeable amount of calcium is found in the medulla. The entire parenchyma is hyaline and necrotic. The cortex is infiltrated with lymphocytes, and is invaded by numerous fibroblasts and capillaries. Several multinuclear giant cells are found in this area. The calcification involves the interstitial tissue as well as tubular and glomerular epithelium and also the walls of the blood vessels. The calices are covered completely by healthy looking transitional epithelium which lines also many of the collecting tubules. Numerous calcific deposits are noticed in the calices and are surrounded by young connective tissue. Lymphocytic infiltration is well marked in this area. These calcific deposits are being invaded by fibroblasts and capillaries, and their edges have become irregular as if an erosion is taking place.

Surrounded by a mass of connective tissue in the proximity of the epithelium of the calyx there is a small spicule of bone whose periphery is lined by irregular layers of fibroblast-like cells, the axis of whose nuclei is chiefly perpendicular to the surface of the bone. Some of the bone cells along the edge of the spicule still retain the shape of the cells which cling to the periphery, while others have assumed an appearance typical of well developed bone corpuscles. No marrow cavity or Haversian system is as yet formed. There is, however, a small dent on the side of the bone in which are found a minute capillary containing a few erythrocytes, a row of fat spaces, and several fibroblasts evidently migrating

from the surrounding connective tissue areas. A multinuclear giant cell of the osteoclast type clings to the edge of the bone.

Animal 6. 32 Days after Ligation.—The macroscopic appearance of the left kidney resembles closely that of the previous animal. The cut surface, however, shows several small bony shells along the pelvis of the kidney.

Microscopic Examination.—Same general appearance of the cortex and medulla as that of the previous animal. The number of spicules of bone is now greatly increased. Lying underneath the epithelium of the calices and separated from it by connective tissue layers of variable thickness, these spicules are at several places directly continuous with a rather dense connective tissue. In most places this connective tissue is separated from the bone by a layer of cells not unlike periosteum, but in other places its fibers merge directly into the bone. If one traces such an osteoid tissue outward into the medulla, one finds that it is continuous with the connective tissue which extends from the capsular regions.

The spicules of bone are perforated by cavities of various sizes, and their edges are made irregular by numerous indentations and projections. These cavities and indentations are lined by a layer of cells closely resembling periosteum or endosteum. Some of these cells are intimately united through their cytoplasm with the matrix of the bone, as if they are being converted into bone corpuscles. Fibroblasts and capillaries occupy these cavities; a few leucocytes of mononuclear as well as polynuclear variety are also found outside the capillaries. Numerous clear spaces encircled by narrow rims of cytoplasm are seen between the fibroblasts, giving rise to an appearance similar to that of reticular connective tissue. Some of the fibroblasts seem to have lost their characteristic tapering protoplasmic processes and appear rounded off. A few multinuclear giant cells with oxyphilic cytoplasm are noted along the edge of the bone.

Animal 7. 33 Days after Ligation.—In this animal a segment of ureter just below the pelvis had been resected at the time of the operation. The gross appearance of the kidney is about the same as in the two preceding animals.

Microscopic Examination.—Practically the same appearance that was found in Animal 6, although the spicules of bone are fewer in number and the epithelium of the calices is thinner. The only two spicules of bone that are found lie under the epithelium and are directly continuous with the fibrous connective tissue which surrounds them. There is a close connection between the connective tissue growing in from the capsule and that proliferating in the calices.

Animal 8. 35 Days after Ligation.—The gross and microscopic pictures are practically identical with those described in the two preceding animals. Five or six centers of bone formation, lying under the epithelium, or around the buds of epithelium which are extending into the medulla, are noted. Most of them show a continuation from the connective tissue to bone as previously mentioned. Several spicules have a boundary layer of osteoblasts (Fig. 1). Cavities in the bone contain a fairly large number of myelocyte-like cells with finely granular, amphophilic cytoplasm and large single, vesicular nuclei of either oval or V shape. Mitosis is rather frequently encountered among the cells that occur within the

cavities of the bone. Coarsely granular eosinophilic forms of such cells also are found. Similar cells are met with in the connective tissue areas near the bone and also at a considerable distance from it. Fibroblasts and dilated capillaries also occur within the cavities. The connective tissue of the marrow now assumes a definitely reticular form.

Animal 9. 35 Days after Ligation.—The gross appearance of the left kidney is similar to that of the previous animal.

Microscopic Examination.—Sections show a great proliferation of buds of epithelial cells extending into the medulla of the kidney. Some of these buds form tubular structures with lumina in the center; others are so small and their epithelium is so flattened that they resemble capillary blood vessels with somewhat thickened intima; all such structures are, however, traceable by means of serial sections to the epithelial covering of the calices.

Spicules of bone are numerous and vary greatly in size. Where there is a dense connective tissue nearby a direct connection can be established between the bone and the connective tissue (Figs. 2 and 3). Many of the spicules lie in a highly cellular connective tissue; in such instances the bone is lined along the periphery by periosteal membrane. Cavities within the bone contain reticular connective tissue in which are suspended young fibroblasts, capillaries, and a few myelocyte-like cells elsewhere described. All the bone spicules lie close under the epithelium of the calices or around the epithelial buds. Degenerating epithelial cells with pycnotic nuclei and swollen granular cytoplasm, frequently containing clear, globular vacuoles, occur in the lumina of the pelvis and epithelial buds.

Animal 10. 35 Days after Ligation.—The left kidney is diminished in size and shows on gross inspection only a narrow layer of calcification under the capsule and a long shell of bone in the calyx.

Microscopic Examination.—The epithelial proliferation is similar in extent to that found in the previous animal. The calcium deposits are confined chiefly to the cortex, but are also found in the medulla. A few narrow but long spicules of bone are located in the proximity of the epithelium of the calices and around the buds of epithelium. They contain cavities which are similar in appearance to those found in the previous animal. The epithelium over the bone appears to be degenerated in many places.

Animal 11. 41 Days after Ligation.—The left kidney is small and shows on the cut surface a dense calcification in the cortex and small, hard foci of either calcification or ossification in the grayish medulla, close to the pelvis.

Microscopic Examination.—A considerable outgrowth of epithelial buds is found. Large spicules of bone are present in the connective tissue areas of the calices, a direct continuation between the bone and the connective tissue fibers being well demonstrated. The cavities in the bone contain the same structures as those found in the two preceding animals.

In areas close to the bone there is an areolar connective tissue which contains in its reticula a rather large number of mononuclear leucocytes of various

shapes and sizes. Most of these cells appear to be ordinary lymphocytes; others resemble myelocytes and possess a large cytoplasm, which takes up eosin stain, and oval or notched nuclei. Calcific placques are found in the neighborhood of the bone. The loose connective tissue described above is continuous with the connective tissue area surrounding the necrotic medulla of the kidney.

Animal 12. 54 Days after Ligation.—The left kidney is very much smaller than the right. It contains dense deposits of calcium throughout, and spicules of bone in the calices.

Microscopic Examination.—The entire renal tissue has been replaced by connective tissue, except in a small area where a trace of glomerular structure is still visible. Calcium deposit is noted practically everywhere in the organ, except in areas close to the pelvis where the connective tissue proliferation is most pronounced. Extension of epithelium of the calices into tubules has taken place, but the epithelial cells have become much lower, the nuclei mostly pycnotic, and the cytoplasm is vacuolated in many instances.

Spicules of bone are numerous and are more widely distributed than in any of the previous animals. In addition to those occurring near the pelvic epithelium there are several foci of ossification in the medulla. In the latter situations many small spicules are being laid down in such a way as to become directly continuous with calcific placques. These placques are surrounded by a large number of fibroblasts, capillaries, and comparatively few lymphocytes. A few fibroblasts are seen clinging to the edge of the calcium deposits, and become incorporated into their periphery, giving rise to an appearance similar to the new bone laid down along the lime plates (Fig. 5). In some instances epithelial buds derived from the calices are noted in the vicinity of these new forming bones; in other instances epithelial structures are nowhere to be seen. One of the bone spicules thus formed in direct contact with calcific placques contains in its cavities strings of fibroblasts, capillaries, and numerous leucocytes of various types. Some of these leucocytes are either lymphocytes or polynuclear neutrophils, but the majority resemble myelocytes. There are also quite numerous giant cells that cling to the edge of the bone. Megacaryocytes are also frequently seen, some of them being found in the act of phagocytosing myelocytes and polymorphonuclear leucocytes.

Near the calices the cavities occur within the substance of the bone or between it and the surrounding dense connective tissue. These cavities contain the same types of leucocytes that were described in the previous paragraph. It is among these myelocytes that mitotic figures are not infrequently observed. Some of the dividing myelocytes possess coarse, granular, eosinophilic cytoplasm, while others contain fine amphophilic granules.

Animal 13. 66 Days after Ligation.—The gross appearance of the left kidney is similar to that of the previous animal. Narrow shells of bone are visible close to the pelvis on the cut surface.

Microscopic Examination.—Sections show many narrow epithelial buds which have grown out into the medulla. A dense connective tissue extending from

the capsule meets that coming from the calices and together forms highly cellular layers around calcific deposits. Only three small spicules of bone are found immediately under the epithelium of the calices.

Animal 14. 71 Days after Ligation.—The left kidney is very much smaller than the right and forms a gray, stony, hard mass.

Microscopic Examination.—The medulla is found to be composed largely of organization tissue containing a great number of epithelial buds growing outwards from the calices. Numerous small spicules of bone are also found.

Animal 15. 94 Days after Ligation.—The left kidney is fairly large and cuts with considerable difficulty. It is completely surrounded by a mass of fat as in all other instances. The gross appearance of the cut surface does not differ materially from that in the previous animals, except that the bone spicules are distinctly larger, with specks of bright red color suggesting red marrow cavities.

Microscopic Examination.—Several large spicules of bone are found at the original site of the calices. Bone cells appear characteristic. Complete Haversian systems are quite numerous, the smaller ones containing only capillaries while the larger ones enclose, in addition, several cells closely resembling myelocytes. These Haversian canals are mostly lined by layers of cells which resemble endosteum. Lamellations of the bone are typical. Some of the Haversian canals may be traced into the larger cavities to be described immediately, and contain capillaries that are directly continuous with those found in the latter cavities.

The larger cavities above referred to lie either within the bone, between the bone and connective tissue, or within the wall composed of a number of smaller spicules of bone. Such cavities resemble bone marrow in several respects. Fat spaces are distributed in the cavities somewhat as in normal marrow, but seem to be less numerous. Between these fat spaces are found masses of leucocytic cells, the majority of which are mononuclear and transitional myelocytes of amphophilic variety. Eosinophilic and basophilic types also occur, but much less frequently. Numerous polymorphonuclear neutrophilic leucocytes and lymphocytes are also observed. Typical normoblasts and megaloblasts are present in small numbers.

There are from six to eight giant cells in each microscopic field, with ocular 4 and objective 3. Some of these possess single, deeply staining nuclei of large size while a smaller number are multinucleated. Several of these giant cells have evidently ingested polymorphonuclear leucocytes and myelocytes in various stages of degeneration (Fig. 4). Erythrocytes are scattered among the marrow cells as well as within capillaries. Mitotic figures occur not infrequently among the myelocytes.

Animal 16. 100 Days after Ligation.—The left kidney is small and is densely infiltrated with calcium deposit throughout, except at the pelvis where there is a soft, grayish white tissue mass. Bright red streaks about 3 mm. wide are noted at the corners of the pelvis and appear to be enclosed within thin shells of bone.

Microscopic Examination.—The cortex consists of masses of calcified tubules with hyaline connective tissue between them. The region of the pelvis contains

also hyaline connective tissue with numerous small round cells and fibroblasts. Near the calices which are lined by thin epithelium there are large numbers of tubules lined by an extension of the epithelium. There are large numbers of bone spicules in the calices, some of them extending so far out that they become directly continuous with calcified tubules of the medulla. In places they are pierced by epithelial tubules created by an extension of the epithelium of the pelvis. The bone cells are identical with those found in normal bone. In the spicules which are lying closer to the marrow cavities, however, some of the bone cells have become indistinct, the osseous tissue forming a deeply eosin-staining mass devoid of definite lamellæ and cell membranes. At several places the osseous tissue passes over gradually into hyaline connective tissue which contains also some young fibroblasts.

The marrow cavities contain the same type of leucocytic cells that were described in the previous animal. Myelocytes, megacaryocytes phagocytosing myelocytes and leucocytes, fat spaces, capillaries, and other constituents of the normal marrow are all present.

Such structures occur also in the connective tissue areas of the medulla and between the bone and the connective tissue. Wherever these structures are found some fragments of bone are usually present.

Animal 17. 104 Days after Ligation.—The gross appearance of the left kidney is similar to that of the previous animal. In the calices are noted a few grayish white shells of bone enclosing red streaks about 3 mm. wide and 1 cm. long.

Microscopic Examination.—The appearance is much the same as that previously described. In this kidney, however, the spicules of bone are slightly more numerous. The cavities that occur within the bone and between the bone and connective tissue resemble closely the normal marrow of the rabbit. Giant cells of megacaryocyte type are fairly numerous and are actively engaged in phagocytosis of myelocytes and polymorphonuclear leucocytes. One or two small sequestra without bone cells or endosteum are found lying among the mass of myelocytes. The edges of the bone facing the marrow cavites are markedly irregular and appear to be eroded, in these places the bone cells being indistinct and the endosteum absent. Masses of leucocytes may be seen piling up along the eroded edges.

Animals 18, 19, and 20.—The left kidneys in these animals are covered by a dense mass of fat. Upon opening this fatty capsule a collection of thick, creamy pus is found exuding from the very small kidney whose tissues have become extremely friable. Neither calcium nor bone is found.

Summary of Histological Findings.

Of twenty rabbits whose renal artery and vein were ligated, three became infected; of the remaining seventeen animals, bone was produced in all but the first four of the series. The presence of bone

became macroscopically evident as early as 32 days after ligation, and the bone continued to become larger and thicker until about the 54th day, when definite red marrow could be recognized. The sequence of events as we have observed it may be summarized briefly as follows:

Hyaline Degeneration.—3 days after ligation the kidney is swollen and dark red. Sections through the organ show a definite hyaline change in the cytoplasm of the renal tissues. The nuclei of the cells are more pycnotic than normally. No caryorrhexis has yet occurred, and the cells although hyaline are not yet completely necrotic. There is a zone of leucocytes close under the capsule. The epithelial covering of the calices is also hyaline and in places is desquamated *en bloc*. The progress of the degeneration of parenchymatous tissue, however, is slow, viable looking epithelial areas being present as late as 70 days after ligation.

Calcification.—A histologically recognizable degree of calcium deposition is observed 3 days after ligation. At this stage, however, it is present only as extremely minute traces of granular deposits and is confined to the tubular and glomerular epithelium along the zone of leucocytic infiltration under the capsule. 14 days after ligation the amount of calcium deposited is only slightly increased over that of the 3rd and 7th day stages and is still confined to the external portions of the cortex. 27 days after ligation the amount of calcification is decidedly increased and the process involves the medulla as well as the cortex. From this stage on until about 41 days after ligation the increase in the amount of calcium, as it is roughly estimated by histological appearance, is rapid and progressive, involving not only the epithelial elements but the interstitial tissue and the wall of the vessels as well. As the calcium salts are continually deposited, relatively large placques replace the formerly granular deposits. These placques are found in various shapes and sizes and occur in the cortex, medulla, and in the areas close to the pelvis. Quite frequently they present irregular indentations along their edges in which are found capillaries and fibroblasts migrating from the surrounding granulating tissue. Multinucleated giant cells also occur frequently in the neighborhood of these lime plates. New connective tissue grows in both from the capsule and from the calices and slowly replaces the necrotic tubules

and glomeruli, and surrounds completely the islands of calcium deposits. No fatty degeneration or infiltration has been noticed prior to calcification. The preliminary step to calcification as we have observed it consists thus of coagulation necrosis following the induction of anemia.

Ossification.—The first evidence of bone formation is noticed 28 days after ligation. From this time on, all the kidneys examined show the presence of more or less bone except in the three animals whose left kidneys had become infected.

The site of the occurrence of bone is consistently the connective tissue areas of the calices close under the transitional epithelium which is similar in appearance to ureteral epithelium. In earlier stages no other areas contain bone, whereas spicules are found well out in the medulla in some of the later stages. In no case, however, is there an evidence of ossification originating under the capsule. The total amount of osseous tissue increases on the whole, and the appearance of the bone becomes more characteristic as time goes on.

As early as 32 days after ligation thin bony shells are visible to the naked eye. By 94 days it is possible to foretell the presence of typical red marrow by observing with the naked eye streaks of bright red areas enclosed by spicules of bone. From 66 days on it becomes gradually more evident that the resorption of bone is more rapid than its formation. Multinucleated giant cells of the type usually spoken of as osteoclasts are more frequently seen in the later stages than in the earlier. These giant cells are closely applied to the periphery of the bone into which they appear to have penetrated to variable distances. At 94 days and thereafter sequestra make their appearance in the marrow cavities; some of the bone spicules become fragmentary and their bone cells less distinct than in the earlier stages; and in many instances the periphery of the spicules is much more irregular than formerly.

The processes of bone formation in the kidneys as we have observed them are as follows: Following the hyaline degeneration and calcification of renal tissues the connective tissue cells take part in bone formation in three different ways. In the first place, rather loose fibrous connective tissue is formed by a proliferation of fibroblasts that accompany the capillaries. The transitional epithelium of the

upper part of the ureter comes up with these structures. In the connective tissue thus formed under the epithelium a small spicule of bone makes its appearance. In general, the axis of bone cells is parallel with that of the surrounding connective tissue cells. An irregular layer of fibroblast-like cells surrounds the bone in a manner similar to the periosteal membrane; some of these cells have become continuous through their cytoplasm with the matrix of the bone, the nuclei still retaining their large vesicular form. A few lymphocytes as well as several loose fibroblasts lie close to the periosteum-like membrane (Fig. 1).

In the second type of ossification, seen frequently but only where the type described above has initiated the process and is still actively at work, there is a direct ossification of the hyaline connective tissue in continuity with the periphery of the preexisting bone (Figs. 2 and 3).

In the third type of ossification, seen only in one specimen in which epithelial buds and bone spicules extend far out into the medulla, the bone is formed directly in contact with the eroded edges of the lime placques. In this case the fibroblasts that erode their way with the capillaries into the calcified tubules seem to take on an osteogenic function and bone is laid down, largely in concentric lamellæ, against the lime deposits. In some places the bone formation begins at the periphery and surrounds a placque. As these various foci of ossification grow they produce coalescing conglomerates of true bone and calcium precipitate, which form large dense spicules, in which very little marrow is seen, though the Haversian canals contain highly vascular reticulum (Fig. 5).

Formation of Bone Marrow.—A slight suggestion of a cavity in the bone is present in the earliest stage at which the bone is found. This consists of a dent in the periphery of the spicule and is occupied by a row of a few fat spaces along the edge of the bone, fibroblasts migrating from the surrounding connective tissue areas, and a minute capillary containing a few erythrocytes. Within this cavity is seen a multinuclear giant cell of the so called osteoclast type clinging to the edge of the bone. Complete marrow cavities may be produced either through an enlargement of a dent in the periphery of the bone together with an ossification of the neighboring connective tissue areas, or

through bone formation around an area of reticular connective tissue elsewhere described. In earlier stages the contents of these cavities consist of fibroblasts, capillaries, and fat spaces. Leucocytes, chiefly small lymphocytes, are also found in them.

At the 35 day stage rather large numbers of myelocyte-like cells are noted. These cells possess a large cytoplasm with fine amphophilic granules and a large, vesicular, single nucleus of oval or V shape. Some containing coarse, eosinophilic granules also occur, but much less frequently. Such cells are found in groups quite apart from bone as well as within the marrow cavities. Whereas in the areas free from bone no mitotic figures can be observed, they are frequently seen among the cells lying within the marrow cavities. Direct evidences of genetic relation between these myelocyte-like cells and fibroblasts or large mononuclear leucocytes cannot be established in our series. Even when they occur away from the existing bone, such areas may later be surrounded by new forming bone. The typical marrow previously described is seen in the bones which are at least 5 weeks old. In the kidneys which contain such marrow, myelocytes are confined strictly to the cavities within the bone or to the close neighborhood of the bone. The mitosis of the myelocytes which was described for the 35 day stage is present even as late as 104 days after ligation. Apparently the abundance of myelocytes in the later stages owes its origin to a rapid proliferation of these cells.

Series 2. Autotransplantation of Cartilages.

In this series fourteen animals were killed from 7 to 80 days after subcutaneous autotransplantation of ear and xiphoid cartilage.

Experimental Data.

Animal 1. 7 Days after Transplantation of Boiled Cartilages.—Both xiphoid and ear cartilages are encapsulated in a very vascular, loose connective tissue.

Microscopic Examination.—The lacunæ in the pale blue matrix contain groups of hyaline cells with pycnotic nuclei. The perichondrium is stained deep red and the nuclei of the cells appear more pycnotic than normally; the inner and outer borders of the perichondrium are sharply defined. No indication of new formation of cartilage is found. The surrounding connective tissue is loose, vascular, and contains large numbers of leucocytes, both polymorphonuclear and small mononuclear.

Animal 2. 10 Days after Transplantation.—The gross appearance of the cartilages is similar to that in the previous animal.

Microscopic Examination.—The matrix of the cartilages is stained deeply with eosin; the cells in the lacunæ are normal in several places, their nuclei somewhat pycnotic elsewhere. The dense perichondrium is infiltrated to some extent with polymorphonuclear leucocytes and to a greater extent with small mononuclear leucocytes, and shows transitional stages of new cartilage formation. In places there are seen stellate groups of eight or ten cartilage cells in a homogeneous matrix deeply stained with hematoxylin. The loose connective tissue surrounding the transplants is richly cellular and is rather densely infiltrated with leucocytes.

Animal 3. 16 Days after Transplantation. Microscopic Examination.—The perichondrium and the surrounding connective tissue appear as in Animal 2, except that the infiltrating leucocytes are more predominantly mononuclear in type. The central part of the cartilage is stained deep red, the nuclei of the cartilage cells are more pycnotic, and their cytoplasm contains vacuoles. On the peripheral layers of the cartilages, especially of the ear cartilage, there are found several layers of newly formed cartilage cells.

Animal 4. 21 Days after Transplantation.—No changes in appearance are noted from that in the preceding animals, except that the leucocytic infiltration is less intense.

Animal 5. 28 Days after Transplantation.—The gross appearance of the transplants is the same as in the previous animals.

Microscopic Examination.—The lymphocytes infiltrating the surrounding connective tissue are few in number. There are several islands of newly formed cartilage separated from the original transplant of the ear cartilage by a small number of layers of perichondrial cells. Similar proliferation does not occur in the xiphoid cartilage.

Animal 6. 43 Days after Transplantation.—Both cartilages form hard masses in a fibrous capsule which is denser but less vascular than that seen in the previous stages.

Microscopic Examination.—Sections of the xiphoid cartilage show nothing unusual, the appearance being similar to that seen in the 1 week stage. The original cartilage appears as a rather faintly stained pink cast with empty lacunæ in many places. Lying on each side of it is a thick growth of new cartilage; that nearest the old cartilage consists of masses of deep blue cartilage cells with dark granules marking the nuclear structures, and separated from each other by a deeply red hyaline substance. Along the borders and out in the perichondrium are layers and islands of young, growing cartilage with a pale blue matrix and lacunæ containing cells with red, finely granular cytoplasm and nuclei which are either vesicular or, as in several places, somewhat pycnotic. The perichondrium merges into the surrounding dense connective tissue.

Animal 7. 59 Days after Transplantation of Boiled Cartilages. Microscopic Examination.—Sections show that most of the cartilages are represented by mere pink shadows with empty lacunæ, although in places there are cells which look

exactly like those in the 7 day stage, suggesting that either the boiling has not been sufficiently long or the cartilage cells have been fixed by being boiled. No newly formed cartilage is, however, noted in the perichondrial connective tissue. In places the lacunæ present a dark-blue-stained lining as though calcification were beginning to take place. In the dense connective tissue surrounding the cartilage a spicule of bone about 0.5 by 1 mm. in size is found. It contains a cavity filled with myelocytes, a few multinuclear giant cells, fat spaces, and reticular connective tissue. Its inner border is lined with a membrane of cells similar to endosteum (Fig. 6). Although it is difficult to rule out a possibility of its connection with cartilage, in all of half a dozen sections taken at various levels we are unable to establish such a connection.

The connective tissue surrounding the bone is rich in young fibroblasts, and the transition from osseous to connective tissue is gradual at one end. The bone cells are laid down in concentric circles around the marrow cavity and the fibroblasts around the bone are also so arranged; thus the bone and the immediately surrounding connective tissue form an eddy-like structure in a mass of fibrous tissue whose fibers run in lines parallel with the axis of the cartilage.

Animal 8. 67 Days after Transplantation. Microscopic Examination.—The sections show a condition about the same as that of Animal 4, except that there is more newly formed cartilage. Careful search shows no signs of ossification.

Animal 9. 69 Days after Transplantation. Microscopic Examination.—The xiphoid cartilage shows no well marked changes. The ear cartilage appears much like that seen in Animal 5. The original transplant appears totally necrotic, although in some places proliferating cartilage can still be seen on one side under the perichondrium. On the border of the dead cartilage there is a layer of pale blue, finely striated hyaline tissue which corresponds to the original perichondrium. Peripheral to this is a very thick layer of new cartilage. In two places bony spicules, 1 to 2.5 mm. in length, extend into the new cartilage from the newly formed perichondrium (Fig. 7). They seem to erode the cartilage and extend as far as the old perichondrium. They contain Haversian systems, marrow cavities, an endosteum-like membrane of cells, and in places incorporate into their substance shadows of cartilage cells.

Animals 10, 11, and 12. 72 Days after Transplantation. Microscopic Examination.—General appearance of sections is much like that found in the previous animals. In Animals 11 and 12 the old cartilage is fairly well preserved, and there is somewhat less growth of new cartilage than in Animal 8. All the newly formed cartilages are found in the form of islets of cells in the perichondrium.

Animals 10 and 11 show two, and Animal 12 shows one spicule of bone. All these bones are located in the perichondrium, and extend from it into the newly formed cartilage, exactly as in normal endochondral ossification. These bony spicules contain typical Haversian systems and are lined along their inner borders by an endosteum-like membrane (Fig. 8).

Animals 13 and 14. 80 Days after Transplantation.—Both specimens are similar to the previous ones in their gross appearance. Like them neither shows any marked changes in the xiphoid cartilage.

Microscopic Examination.—In both instances the original transplants of ear cartilage show definite evidences of degeneration, and there is definite growth of new cartilage from perichondrium. Animal 13 shows a round spicule of bone towards one end of the cartilage. The relation of connective tissue to bone is similar to that found in Animal 7.

Animal 14 shows a round spicule of bone near the ear cartilage transplant larger than the one just described. This spicule is surrounded by a thin layer of connective tissue on three sides so that it has the appearance of projecting from the perichondrium into an area of areolar connective tissue.

Summary of Histological Findings.

In the fourteen animals in which the ear and xiphoid cartilages were autotransplanted, bone was found in seven specimens of ear cartilages removed from 59 to 80 days after operation. One of these ear cartilages had been boiled previous to implantation. In xiphoid cartilages transplanted and removed under the same conditions and in the same animals as corresponding ear cartilages, bone formation did not take place.

Reaction of Tissue around the Transplant.—In the connective tissue around live or boiled transplants a definite inflammatory reaction is seen in the 7 day stage; polymorphonuclear and lymphocytic infiltration, an increase in the capillaries, and a proliferation of fibrous connective tissue cells are evident. This inflammatory reaction subsides gradually as seen in the 16, 21, and 28 day stages. The connective tissue surrounding the transplants blends completely with the perichondrium by the 43rd day, and makes it appear possible that it has taken up the function of perichondrium.

Changes Occurring in the Cartilage.—In the early stages the gradual degeneration and necrosis of the original cartilages can be followed. Beginning at the center the matrix assumes an eosin tint and the nuclei become pycnotic; the peripheral layers of the cartilage and the perichondrium appear healthy at earlier stages. The specimen of boiled cartilage removed in earlier stages shows practically the same general picture, except that here the perichondrium is entirely necrotic and only the matrix of the cartilage appears normal; the cells of the periphery retain their form and the nuclei stain deeply in hematoxylin. The general impression gained from such a picture is that the boiling may perhaps have fixed the cells. With the ear cartilage the periph-

eral layers seem to be actively proliferating, the new cartilage cells being formed from the perichondrium. In the case of the xiphoid cartilage, on the other hand, no change is seen after this time, the central portions remaining eosinophilic; the peripheral layers and the perichondrium are alive in many places, but no new cartilage is formed even in the later stages. In the ear cartilage the proliferation of new cartilage becomes evident as early as the 16 day stage and it increases as time goes on, although the proliferation is more pronounced with large than with small transplants. In some of the specimens of the ear cartilage removed from the 43 to 69 day stages in which large pieces of cartilage folded upon themselves had been used, the folds of old cartilage now entirely necrotic with empty lacunæ and faintly pink matrix can be traced through the whole section. On each side of this necrotic cartilage, and separated from it in some places by a layer of pale blue hyaline tissue representing the original perichondrium, are seen very thick layers of new cartilage with pink cytoplasm and nuclei varying in appearance from vesicular to pycnotic. The tissue adjacent to the healthy perichondrium resembles normal hyaline cartilage, but in the deeper layers the lacunæ are large and distorted and the matrix is stained deep blue. The general picture almost resembles that of a rapidly proliferating chondroma with the centers necrotic and calcified.

Ossification.—The earliest stage at which bone is found is the 59 day stage in the animal which received an implantation of boiled cartilages. In this animal there is very little evidence of new cartilage formation; the old cartilage appears dead in most places, although areas are found which suggest that possibly some of the peripheral cartilage cells are still alive, and in these areas a dense connective tissue which resembles perichondrium has been formed close around the cartilage. The spicule of bone consists of concentric lamellæ about an endosteum-lined cavity containing a typical marrow. The spicule itself is surrounded by a dense connective tissue whose fibers also run in concentric rings (Fig. 6). In places the bone and the connective tissue merge gradually into each other. A direct connection of the bone and the cartilage cannot be found in this specimen, but this fact does not signify the independence of the bone of any association with the cartilage, since our serial sections have not been complete.

Of the six animals with live cartilage transplants examined from the 69 day stage on, bone is found in all. In the two animals in which only a moderate amount of perichondrial cartilage formation occurred, the spicules of bone are similar in appearance and location to the spicule just described, although both are larger and contain less marrow. In the four animals in which an extensive new formation of cartilage took place with subsequent atrophic changes and calcification as previously described, from one to two bony spicules are found in each specimen, laid down immediately under the perichondrium and invading the calcified new cartilage. In no instance does the ossification occur in the original transplant, nor do the cartilage cells play an active part in the process, although lacunæ may be incorporated in the line of advancing bone formation. The cells of the perichondrium may be so closely associated with the bone cells that a distinction between the two often becomes difficult. Here, as in other instances, the fibers of the connective tissue about the bone are arranged like whorls around the peripheral borders of the Haversian systems. Where invasion of the new cartilage by the new forming bone can be demonstrated, the picture is exactly that of normal endochondral ossification (Figs. 7 and 8). The cartilage cells beyond the advancing lines of ossification show evidences of rapid proliferation, with subsequent degenerative changes and calcification of the matrix. The bone spicules contain marrow cavities rich in capillaries and are lined with endosteum. The borders of these spicules invade the calcified cartilage and in places incorporate shadows of matrix and lacunæ.

DISCUSSION.

Before discussing the new points brought out by the experiments it will be well to consider to what extent we have been able to corroborate the observations and views of previous investigators. Unlike Liek, who used a method similar to ours in studying the rabbit kidney, we did not find evidences of bone formation as early as 16 or 20 days after ligation of the renal vessels. This failure may have been due to differences in the rate of restoration of vascularity. Nor did we observe in the early stages of bone formation in the kidney the close juxtaposition of the bone and the calcium deposits noted by this

author. On the contrary, in our earliest stage the bone was located under the epithelium of the pelvis farthest from the calcified tubules. In all our kidneys prior to the time when the epithelium of the calices had grown out to the lime placques, no bone was found in the cortex.

As to the mode of transformation of connective tissue into bone, two distinct processes have been hitherto described by numerous writers. According to their views, either a hyaline connective tissue or scar tissue may be directly converted into bone, or calcified materials may be eroded with a formation of vascular areas containing young connective tissue cells, some of which take on the function of osteoblasts. As has been described previously in this paper, the histological pictures presented by our specimens probably represent three different methods of transformation. The first and most frequent is the accumulation of young fibroblasts in the area under the transitional epithelium to form a sort of membrane which lays down bone. The bone increases in size by progressive ossification with inclusion of cells of the periosteum-like membrane. Second in frequency is the type described by Lick, Rosenstein, Pearce, and others, in which there is a direct transformation of hyaline connective tissue into bone. This process takes place only in the presence of preexisting bone, and for this reason cannot be considered as a primary process. The third type, in which cells of the vascular granulating tissue which erodes calcium deposits become converted into osteoblasts, was observed only in one of the thirteen specimens showing bone formation.

The theory that heteroplastic bone is formed as a result of stimulation of young fibroblasts by lime salts rests mainly upon the recorded fact that the bone formation takes place in the immediate vicinity of the calcium deposits, since Lick and many others have been unable to confirm the statement made by Barth (16) that injection of calcium salts or implantation of dead bone will stimulate bone formation. In our series of calcium injection, the experimental data of which are omitted for the sake of brevity, no evidence of bone formation was observed as late as 50 days after the injection. In pathological calcification followed by ossification there may perhaps be other factors involved than the mere presence of calcium salts, and these as yet undetermined factors may be essential in the heteroplastic bone

formation. It seems evident that in a richly vascular tissue, cells a few millimeters away from the relatively insoluble salts would be bathed by a tissue fluid of practically the same composition as that found in any other part of the body. A close proximity between the bone and lime salts has not been found in our series. However, it is impossible to exclude the possibility that the young connective tissue cells may have received a stimulus from the calcium salts at the time they first migrated into the necrotic areas poor in blood supply, with the subsequent formation of bone when vascularity was restored and the area again comparatively free from calcium.

In the case of ossification in the cartilage series in which endochondral bone formation was observed, evidence points towards the stimulation of fibroblasts by almost direct contact with the calcified matrix of the cartilage. In the instances in which connection between cartilage and bone could not be established and the process of ossification resembled that in the kidney described as the first type, a stimulation by calcium may have played a part, since as Wells and Benson (17) have shown even the cartilages that do not ossify have some affinity for calcium and absorb the salts from the tissue fluids. It is not improbable that fibroblasts coming in contact with such cartilage might be caused to differentiate into osteoblasts under favorable conditions.

CONCLUSIONS.

1. Bone formation in the rabbit kidney with ligated vessels takes place (*a*) through the activity of young fibroblasts which accumulate to form a membrane-like structure; (*b*) subsequently by direct ossification of hyaline connective tissue in continuity with preformed bone; and (*c*) through erosion of lime placques by granulating tissue and laying down of lamellar bone by cells derived from fibroblasts.

2. Bone formation in the rabbit kidney begins not in direct contact with calcium deposits, but in the loose vascular connective tissue close under the transitional epithelium of the calices.

3. With autotransplanted ear cartilage of the rabbit there is an active new formation of cartilage in the connective tissue which surrounds the transplants, and the bone is formed by the fibroblasts from the perichondrium which erode and invade the calcified areas in this new cartilage.

4. The process of bone formation in the kidney is similar to that found in normal membranous ossification, while with the transplanted ear cartilage the process is identical with endochondral ossification.

We wish to express our appreciation to Dr. E. L. Opie for many courtesies extended to us in connection with this work.

BIBLIOGRAPHY.[2]

1. Asami, G., *Am. J. Med. Sc.*, 1920, clx, 107.
2. von Hansemann, quoted by Buerger and Oppenheimer (6), p. 363.
3. Pollack, K., *Virchows Arch. exp. Path.*, 1901, clxv, 129.
4. Mönckeberg, J. G., *Virchows Arch. exp. Path.*, 1902, clxvii, 191.
5. Bunting, C. H., *J. Exp. Med.*, 1906, viii, 365.
6. Buerger, L., and Oppenheimer, A., *J. Exp. Med.*, 1908, x, 354.
7. Rosenstein, P., *Virchows Arch. exp. Path.*, 1900, clxii, 100. Nicholson, G. W., *J. Path. and Bact.*, 1917, xxi, 287.
8. Sacerdotti, C., and Frattin, G., *Virchows Arch. exp. Path.*, 1902, clxviii, 431.
9. Liek, E., *Arch. klin. Chir.*, 1908, lxxxv, 118.
10. Poscharissky, J. F., *Beitr. path. Anat.*, 1905, xxxviii, 135.
11. Maximow, A., *Anat. Anz.*, 1906, xxviii, 609.
12. Pearce, R. M., *J. Med. Research*, 1909, xx, 53.
13. Harvey, W. H., *J. Med. Research*, 1907–08, xvii, 25.
14. Thoma, R., Text-book of general pathology and pathological anatomy, translated by Bruce, A., London, 1896, i, 499, quoted by Buerger and Oppenheimer (6).
15. Marchand, in Eulenburg, A., Real-Encyclopädie, Vienna and Leipsic, 2nd edition, 1885, i, 693.
16. Barth, A., *Beitr. path. Anat.*, 1895, xvii, 65.
17. Wells, H. G., and Benson, R. L., *J. Med. Research*, 1907–08, xvii, 15.

EXPLANATION OF PLATES.

PLATE 98.

FIG. 1. Bone formation in the rabbit kidney, showing a cell membrane between osseous and fibrous tissue. 35 days after the ligation of the vessels.

FIG. 2. Bone formation in the kidney, showing gradual transition from fibrous to osseous tissue. 35 day stage.

---

[2] Since this article was submitted for publication, a paper by Mauclaire has appeared, summarizing the work on cartilage transplantation (Mauclaire, *Presse méd.*, 1920, xxviii, 545). He states that Zaworikine (1898), Saltykow (1900), and Giani (1911) observed ossification in transplanted ear cartilage.

FIG. 3. Bone formation in the kidney, showing continuity between osseous and fibrous tissue. Weigert-Van Gieson stain. 35 day stage.

FIG. 4. Megalocaryocyte engulfing myelocytes. From the marrow in the bone of a kidney removed 94 days after ligation.

PLATE 99.

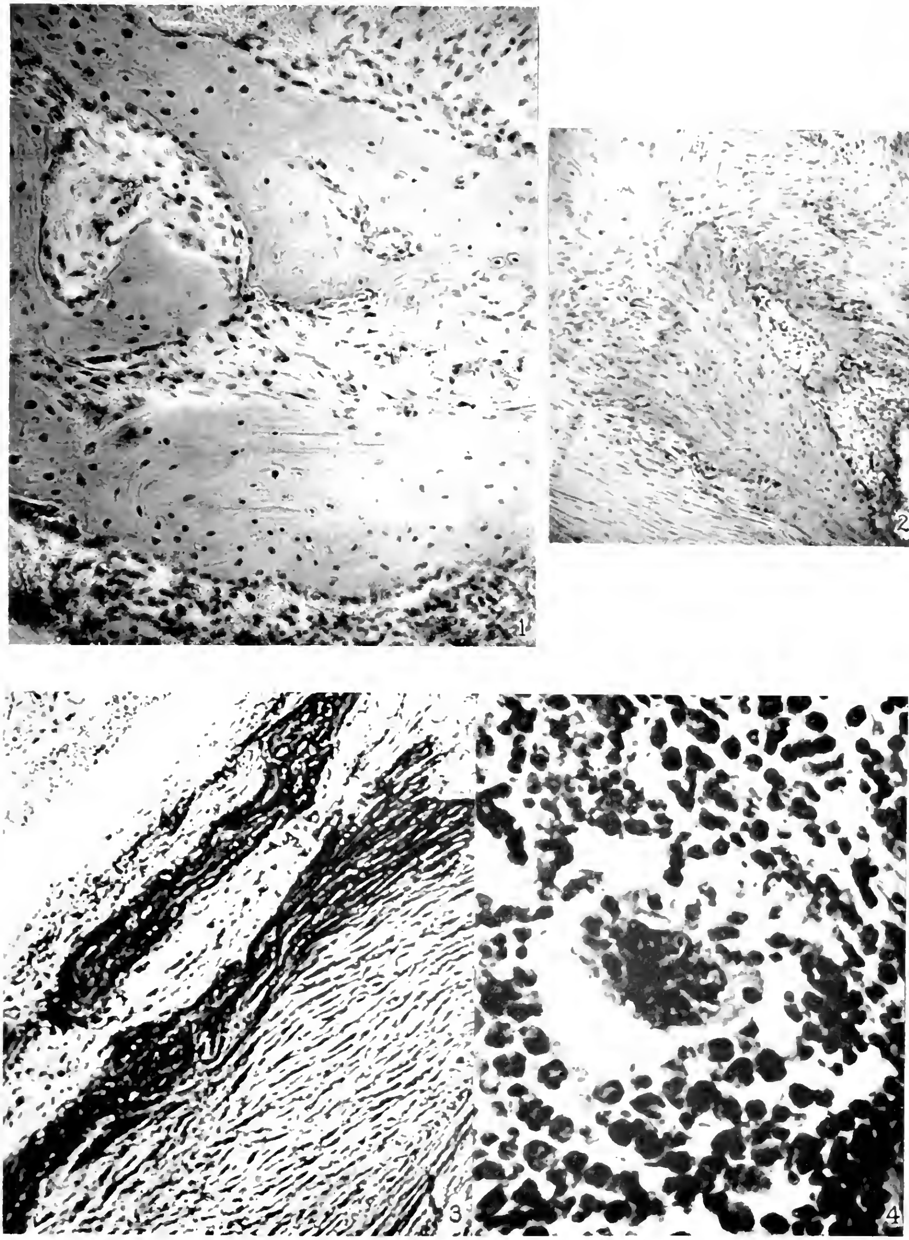
FIG. 5. Bone formation in the kidney, showing Haversian systems forming among eroded calcified tubules. 54 day stage.

FIG. 6. Bone formation with transplanted cartilage. Spicule of bone with marrow cavity and megalocaryocytes. 59 day stage.

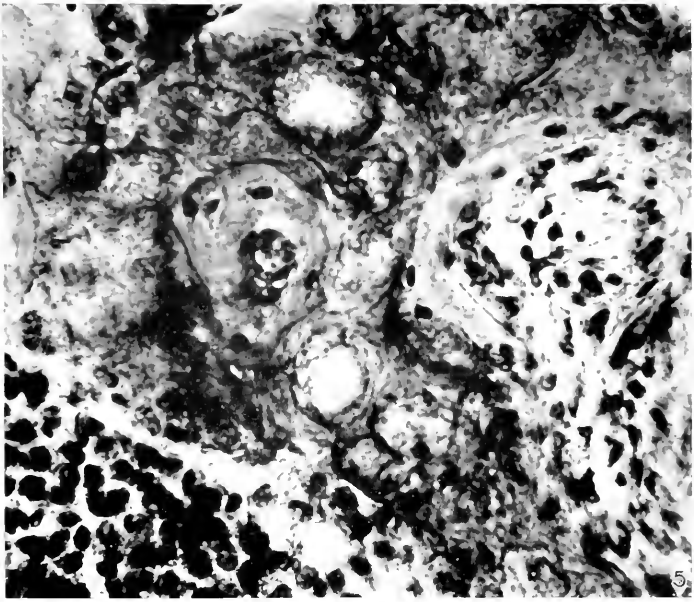
PLATE 100.

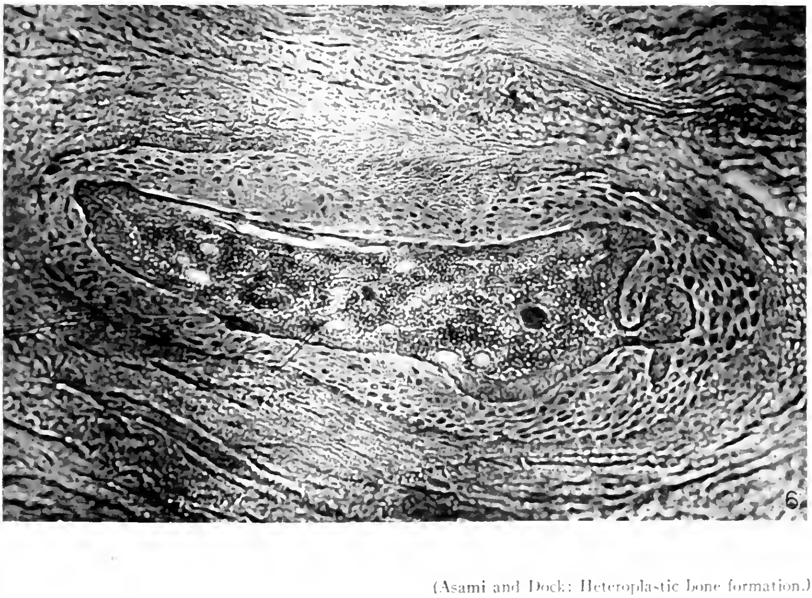
FIG. 7. Bone formation in an ear cartilage transplant. At the bottom are seen the hyaline matrix and cells of old cartilage. Above is new cartilage growth, with beginning invasion by capillaries and osteoblasts. 69 day stage.

FIG. 8. Bone formation in a cartilage transplant. On one side are seen hyaline cartilage cells. The rest of the figure shows Haversian systems forming under the perichondrium. 72 day stage.

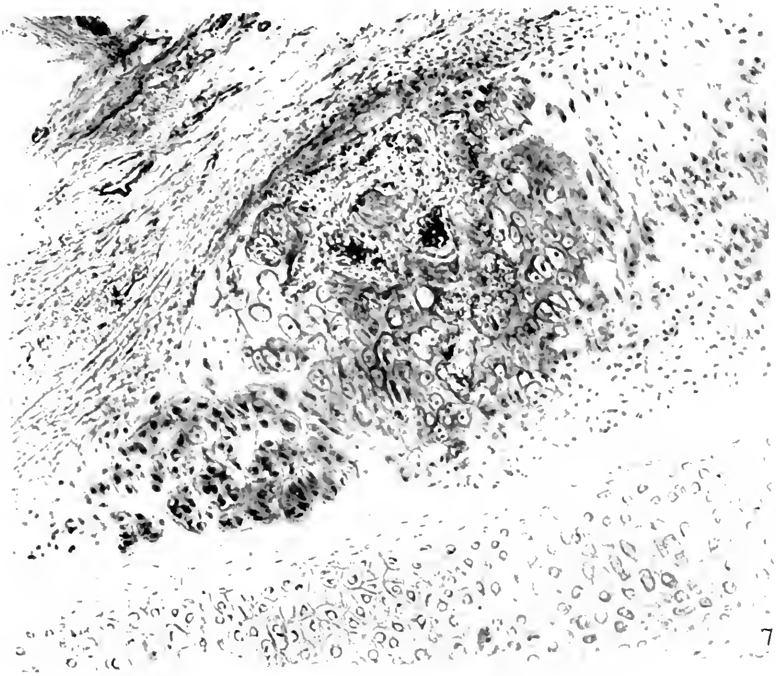


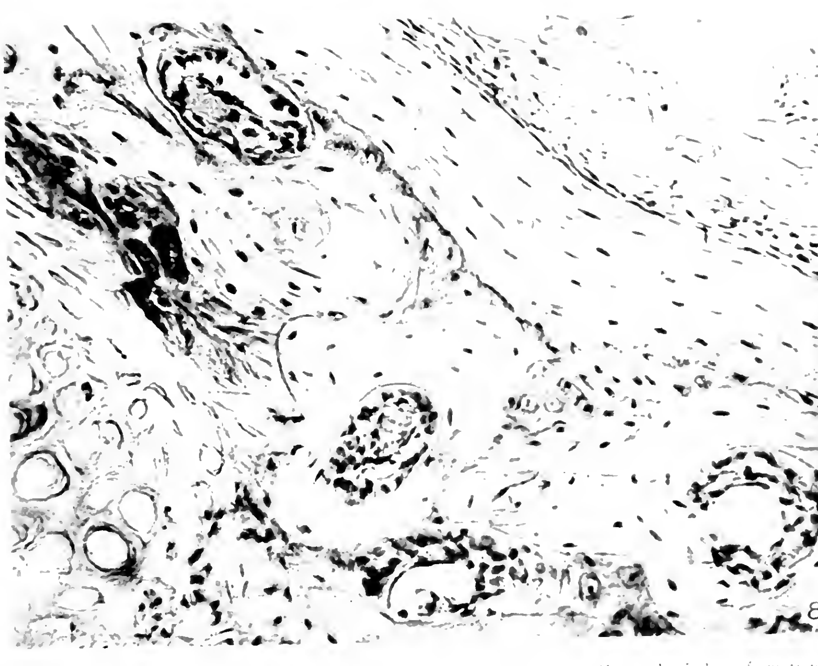
(Asami and Dock: Heteroplastic bone formation.)





(Asami and Dock: Heteroplastic bone formation.)





Asami and Dock: Heteroplastic bone formation.

SIMPLIFIED PRODUCTION OF ANTIMENINGOCOCCIC SERUM.

By HAROLD L. AMOSS, M.D., FREDERICK L. GATES, M.D., and PETER K. OLITSKY, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 10, 1920.)

Although a large amount of work in several countries has been done relative to the production of an effective antimeningococcic serum, the problems involved have not yet been wholly resolved. The increased demands arising from war conditions acted as a potent stimulus first to simplify manufacture and then to insure an effective product. This paper records primarily attempts to simplify the manufacture of an efficacious serum and deals incidentally with a number of still mooted questions regarding the antigenic properties of the meningococcus on which the production of such a serum largely rests.

It is now generally recognized that the meningococcus is not a simple, fixed antigenic entity but rather that the term meningococcus covers a class of closely related microorganisms, the distinguishing common characters of which relate to certain cultural and fermentative qualities and the power to set up in man particular forms of inflammation of the leptomeninges, while they differ markedly in their immunologic responses. Thus for identification the cultural properties are of first importance and for serum production the antigenic structure is paramount.

Ever since Dopter[1] first distinguished the immunologically distinct parameningococcus the classification of the meningococci has been under discussion and agreement has not yet been reached. Everyone admits the existence of two main groups called normal or regular meningococcus and parameningococcus; the disturbing factor is the

[1] Dopter, C., *Compt. rend. Soc. biol.*, 1909, lxvii, 74. Dopter and Pauron, *Compt. rend. Soc. biol.*, 1914, lxxvii, 231.

occurrence of intermediate cocci which resemble either the regular meningococcus or the parameningococcus but are immunologically less sharply defined than are the pure types of the main groups. This fact has led to the setting up of main and subsidiary types as, for example, in Gordon's classification which recognizes four types of meningococci.[2] But Gordon's classification has not received general acceptation and for the reason that observers cannot agree on the two subsidiary type strains.

These considerations have far more than theoretical interest, since experience has shown that a therapeutically effective antimeningococcic serum should possess wide capacities of immunologic activity as measured by the agglutinin content. Since the early work on the subject by Flexner and Jobling[3] and the later studies of Amoss and Wollstein,[4] it has been the custom to employ for the immunization of horses a large number of cultures including representatives of the regular, the para, and many intermediate strains of the meningococcus. The number of strains used in the antigen might reach 50 or more, depending upon the reaction of the serum with meningococci derived from the cerebrospinal fluid of many cases of epidemic meningitis. When the test with such a culture showed low agglutination titer the strain was added to those used for immunization. The purpose of this was to produce a serum with as wide an agglutination index as practicable.

It is obvious that this procedure implies an empirical method. In endeavoring to simplify the manufacture of antimeningococcic serum it seemed worth while to determine more precisely than had yet been done whether a wide agglutination index could be obtained from a small number of antigenically different strains. Several facts had already rendered it probable that something in this direction was achievable. For example, it had been noted that the immunity response in the horse was wider than in the rabbit and also that the longer the injections of certain fixed cultures were continued, the more inclusive the agglutinin content became. In addition to these essential data there

[2] Gordon, M. H., *Med. Research Com., Nat. Health Insurance, Special Rep. Series, No. 3*, 1917, 10.

[3] Flexner, S., and Jobling, J. W., *J. Exp. Med.*, 1908, x, 141.

[4] Amoss, H. L., and Wollstein, M., *J. Exp. Med.*, 1916, xxiii, 403.

was a still further important fact; namely, that approximately 80 per cent of all cases of epidemic meningitis arose from infection with the two type strains; that is, the regular meningococcus and the parameningococcus. Hence the production of an effective serum for this large proportion of cases of meningitis appeared to be relatively simple; the real problem was to make the serum effective against the 20 per cent of cases due to the highly variable subsidiary strains.

Fortunately, we possessed as a standard of comparison for the sera to be produced with a limited number of strains, samples of polyvalent sera, of established therapeutic efficacy, which had been produced at The Rockefeller Institute by the injection of 51 spinal cultures of the meningococcus selected in accordance with the method outlined above.[5] These 51 cultures were classified as follows: 10 were regular, 26 were para, and 15 were intermediate (subsidiary) strains of meningococcus.[6]

Horse Sera Produced with a Small Number of Strains.

When the experiments with horses with a smaller number of cultures of the meningococcus were begun in 1917 we had already observed that rabbits which were immunized with a single type strain over long periods of time yielded sera which contained agglutinins not only for that strain but also, in less amount, for heterologous strains including even those of the opposite type.

Use of Five Strains as Antigen.—The experiments described below were made on two horses (Nos. M 24 and M 25). Injections were begun with four cultures which were classed immunologically as

[5] The polyvalent serum issued for therapeutic purposes was composed of serum from at least three horses. Because of the variation in the response of different horses, it has been our practice to pool serum from several horses to insure a properly balanced product.

[6] In 1914 two strains of parameningococcus were brought from Dopter by a member of the staff of The Rockefeller Institute. It was on the basis of these strains that our classification was made. Recent comparison of our stock strains with sera and cultures lately obtained from England and France shows that our original classification is the reverse of the present accepted grouping. Conforming to general usage, we have revised our classification in this paper, so that the regular or normal group mentioned in previous papers from these laboratories is here designated as para and *vice versa*.

follows: Culture 1, para type strain; Culture 60, regular type strain; and Cultures 30 and 31 of different intermediate types. Serum obtained in early bleedings of these horses did not agglutinate Culture 38 (also an intermediate) which was then added to the antigen.

Experiment 1.—The method of immunization was as follows: After a glanders test and the subcutaneous injection of 1,500 units of antitetanic serum, Horses M 24 and M 25 were tested for sensitiveness to the meningococcus by the intravenous injection of minute doses (0.05 cc.) of a saline suspension (2.5 cc. to an agar slant culture). The immunizing injections were begun on November 29, 1917, living cultures being given in a total dose of 0.12 cc., and were continued according to the method described by Amoss and Wollstein.[4] Trial bleedings were made with Horse M 24 after 3, 10, and 14 months, and with Horse M 25 after 2, 10, and 14 months.

TABLE I.

Number of Stock Strains Agglutinated by the Serum of Horses M 24 and M 25 Immunized with Five Strains of Meningococcus.

Horse No.	Duration of immunization.	Agglutination test positive.	
		1:400 or higher.	1:100
	mos.		
M 24	3	35 of 51	50 of 51
" 24	10	43 " 60	59 " 60
" 24	14	51 " 56	56 " 56
" 25	2	35 " 58	40 " 58
" 25	10	32 " 60	58 " 60
" 25	14	47 " 56	56 " 56

In Table I the progress of the immunization is summarized according to the number of stock strains (cultures) which were agglutinated in a serum dilution of 1:100 and of 1:400 or higher. 51 of these stock strains were those used in the manufacture of the therapeutic polyvalent serum.

Table I indicates that by employing for purposes of immunization as few as five cultures, representing different strains, of the meningococcus, a serum is produced which shows considerable agglutinative capacity for as many as 51 selected stock cultures, including the

English type strains.[7] The titer of the serum for this large number of cultures equalled that previously required for a polyvalent serum prepared with a far larger number of cultures.

Use of Three Strains as Antigen.—In this experiment the strains were reduced to three, representing regular and para types.

Experiment 2.—The procedure with Horse M 31 was similar to that already described. The injections were begun on May 1, 1918, and trial bleedings were made after 5 and 9 months of immunization. Two regular cultures (Strains 60 and 79) and one para culture (Strain 85) were employed for the injection.

Table II summarizes the results obtained, which are the equivalent, for the same period of injection, of those obtained with the five strains as shown by Table I. Although thirteen of the stock cultures did not agglutinate in the 1:400 dilution after 9 months of immunization all were agglutinated in dilutions ranging from 1:50 to 1:200.

TABLE II.

Number of Stock Strains Agglutinated by the Serum of Horse M 31 Immunized with Three Strains of Meningococcus.

Duration of immunization.	Agglutination test positive.	
	1:400 or higher.	1:100
mos.		
5	32 of 50	47 of 50
9	43 " 56	55 " 56

Monovalent Horse Sera.

The total amount of meningococcic antigen which can be given a horse varies and is obviously limited. The use, therefore, of a single antigen might result in a greater antibody content for the homologous strain than could be obtained against the individual strains of a multiple antigen. Indeed, certain therapeutic monovalent sera have been prepared in this way by the Pasteur Institute and more recently by Gordon and others in England working under the Medical

[7] The English type strains were kindly supplied by Dr. Gordon.

Research Council.[8] The intent in both these instances was to fortify the therapeutic activity of the serum for treating cases of epidemic meningitis with type antisera after the type of infecting meningococcus had been determined.

It is not our intention in this place to discuss the practicability of this method of procedure or even to analyze the results thus far secured by the English investigators mentioned above. We would remark, however, that we are dubious as to the advisability of making a substitution of a monoserum for an active polyserum in view of the uncertainties and lack of uniformity which still surround the practical work of determining and distinguishing the so called types of meningococci. But as the data which follow show, the antigenic constitution of even type meningococci is such that a strictly monovalent serum is not produced when horses are injected with a type culture over a long period of time.

The experiments on monovalent sera were made in two ways: first, by injecting as many as three cultures of the same type of meningococcus, and second, by injecting a single type culture only.

Experiment 3.—Horse M 30, after the usual preliminary treatment, was first injected with suspensions of living parameningococci (Cultures 1, 4, and 36). After 6 and 10 months of immunization trial bleedings were made. The serum thus obtained was titrated against 56 stock cultures of meningococci with the results shown in Table III.

TABLE III.

Number of Stock Strains Agglutinated by the Serum of Horse M 30 Immunized with Three Strains of Parameningococcus.

Duration of immunization.	Agglutination test positive.	
	1:400 or higher.	1:100
mos.		
6	40 of 52	50 of 52
10	56 " 56	56 " 56

The results were striking and unexpected, and yet they confirmed certain tests which we had previously made on the variations in

[8] Hine, T. G. M., *Privy Council, Med. Research Council, Special Rep. Series. No. 50,* 1920, 176.

agglutinogenic activity exhibited by different strains of the meningococcus. It appears that in this respect separate strains of the meningococcus show a wide variation. It has been noted also that horses vary in their response (*cf.* Nos. M 24 and M 25, Table I).

The next step was to determine the effect of immunizing horses with single type strains of the regular meningococcus and the parameningococcus. For this purpose two horses were employed.

Experiment 4.—Horse M 32 was injected with a para culture (No. 1) and Horse M 33 with a regular culture (No. 60). The immunization was begun on December 5, 1918, and test bleedings were made 3 months later at a period still too early to show the final wide range of agglutinative capacities of the sera. However, the serum of Horse M 32 (para) agglutinated in dilutions of 1:400 or higher 52 of the 56 stock cultures, and the serum of Horse M 33 (regular) agglutinated in the 1:400 dilution or higher 51 of the 56 stock cultures.

These results raise certain very pertinent questions which cannot be answered offhand. We have dealt with certain aspects of these questions in a later section of this paper in connection with the changes which take place in the sera during storage and the response of the sera produced in different ways to selective absorption tests. We possess many observations which point to the greater efficacy of a truly polyvalent antimeningococcic serum in practice, and hence we do not accept, as yet, such an apparently polyvalent serum, arising from single type cultures, as being immunologically and therapeutically equivalent to the former. The results given show also that an immune horse serum cannot be used for classifying meningococci, and finally, that the so called monovalent sera prepared in France and England in the horse doubtless possessed agglutinative capacities far wider than is implied in their names.

Comparative Agglutinin Content of Monovalent and Polyvalent Sera.

The experiments on horses with single and with several strains of meningococci have shown that agglutinin formation is induced not only for the strain or strains injected but also for a wide number and diversity of other type and subsidiary strains. At first sight it may appear that the sera produced with single and with multiple strains are practically identical and could be substituted for each other in

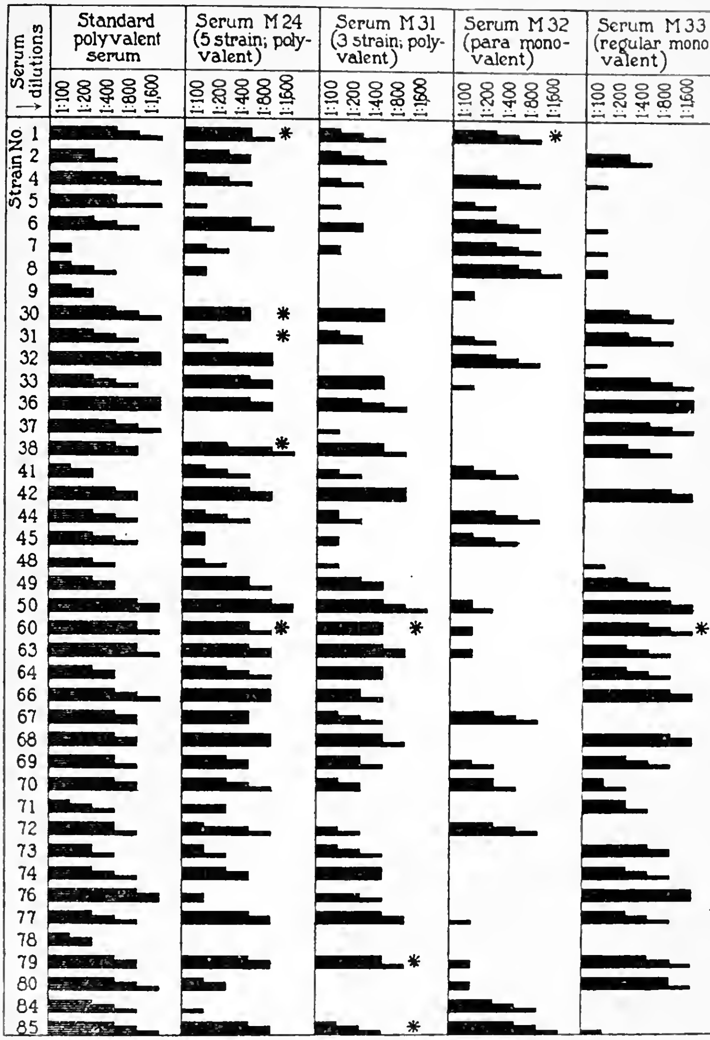
treatment. And yet this deduction would not be justified, as the following tests show. It is also in conflict with observations on the efficacy of widely polyvalent antimeningococcic serum known to contain specific agglutinins for the main and subsidiary strains of the meningococcus.

Effects of Storage.—The first tests to be described here relate to the comparative keeping qualities of the two kinds of sera. The samples of mono- and polyvalent horse sera were kept in the refrigerator in the dark at an approximate temperature of 4°C. for about 1 year. All the sera had been preserved with 0.15 per cent tricresol. The sera tested were derived from Horses M 32 (monovalent para), M 33 (monovalent regular), M 24 (regular, para, and three intermediates), and M 31 (three regular). The polyvalent serum used for comparison was obtained by pooling the serum of six horses, each immunized over a long period of time with 50 odd strains.

The tests of keeping qualities took into account only the agglutinins, which can be quantitatively determined. The titrations were made with suspensions of killed cultures which were uniform for all the tests.

The results of the tests are shown graphically in Text-fig. 1. While the polyvalent serum has fallen off but little during a year's storage and still agglutinates all of the 41 strains of meningococci employed, the two monovalent sera have lost agglutinating power for a considerable number of the strains (sixteen in the case of Horse M 32 (para type) and ten in Horse M 33 (regular type)). On the other hand, the three strain serum (Horse M 31) and especially the five strain (Horse M 24) approach in value the polyvalent serum. The serum of Horse M 24, in a dilution of at least 1:50, agglutinated 39 of the 41 strains.

The chart brings out the important fact that the common or secondary agglutinins are the first to disappear from the serum and that specific agglutinins for homologous strains are reasonably stable during storage. This is a point of capital distinction and may well prove to be the determining factor in respect to the manufacture as well as to the therapeutic efficacy of the various sera. The striking difference as shown by the pooled polyvalent serum relates to its inclusiveness for essentially all the strains employed in the test. It



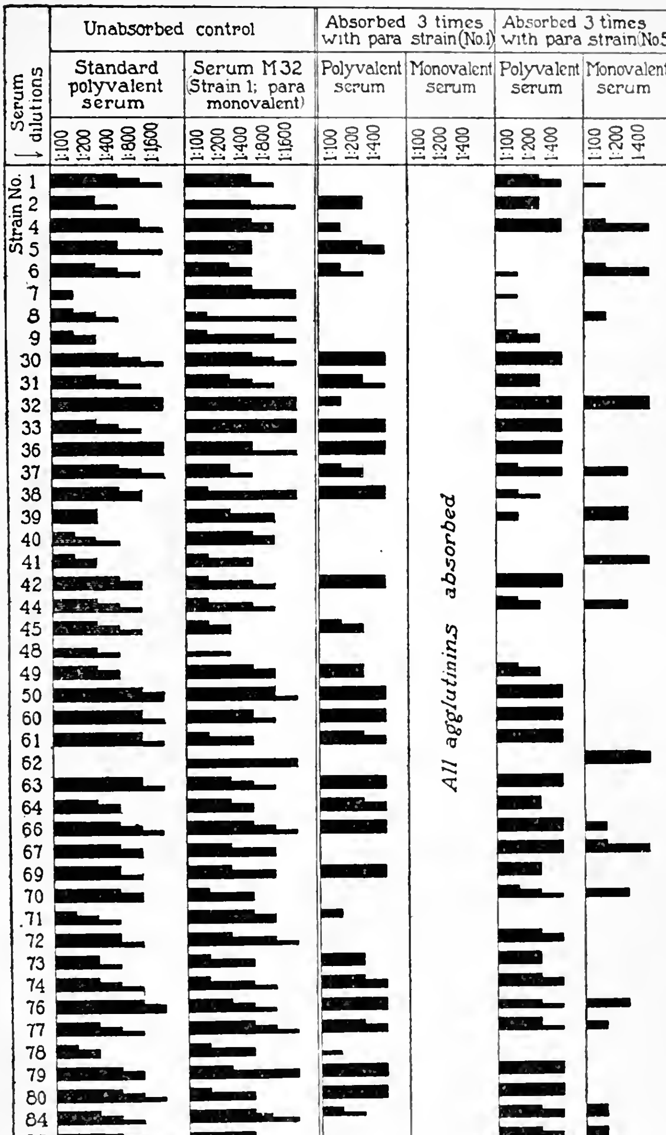
TEXT-FIG. 1. An agglutination test with five sera after storage for 1 year or longer in the ice box. In four of the sera asterisks indicate homologous strains. Complete agglutination in any dilution is represented by a broad black band and indicates the flocculation of all organisms, leaving a clear supernatant fluid, even after shaking. Incomplete agglutination, represented by a band of medium width, indicates the flocculation of almost all the organisms, the supernatant fluid remaining hazy after shaking. Partial agglutination, represented by a narrow band, indicates the presence of distinct flocculi which do not disappear on shaking, although the supernatant fluid is cloudy with unagglutinated organisms.

is obvious that as the monovalent sera lose their secondary (or common) agglutinins they become more suitable for use in type diagnosis, and *pari passu,* more unsuitable for therapeutic purposes. One further matter may be pointed out. The standard polyvalent serum was prepared by pooling the sera derived from six horses. The sera prepared with three and five strains respectively came each from one horse. Reasoning by analogy and taking into account the response of different horses to injections of various strains of meningococcus, for example, Horses M 24 and M 25, it is quite possible that the pooling of sera prepared from a small number of type strains might yield an enduring and still more inclusive serum.

Effects on Absorption of Agglutinin.—The differences in deterioration on storage point to a fundamental difference in the monovalent as compared with the polyvalent sera which is confirmed by the test for absorption of agglutinin as is shown graphically in Text-fig. 2.

In Columns 1 and 2 are shown the control agglutination tests made with a 14 month sample of the pooled polyvalent serum and a fresh sample of monovalent para serum (Horse M 32) before absorption with killed cultures of Strains 1 (the homologous para strain) and 5 (also of para type). After triple absorption under carefully controlled conditions, it was found that the single strains had removed all the agglutinins with which they could react, since further absorption did not reduce significantly the agglutinins remaining in the serum.

Text-fig. 2 shows that Strain 1, the homologous strain, exhausted the monovalent serum completely, but was unable to exhaust the polyvalent serum, leaving agglutinins with which 30 of the 44 test strains were able to react. Similarly, while Strain 5, another strain of the same type, removed from the monovalent serum all the agglutinins with which 27 of the 44 test strains had reacted in the control tests, a similar treatment of the polyvalent serum left available agglutinins for all but 9 of the 44 test strains. This experiment emphasizes the difference in the inherent character of the polyvalent and the monovalent serum.



TEXT-FIG. 2. A comparison by agglutination of standard polyvalent serum and of para monovalent serum before and after absorption three times with a heavy suspension of Strain 1 or 5.

Employment of Killed Cultures.

The preparation of therapeutic meningococcic serum with living antigen is laborious and difficult. Because of the rapidity with which the meningococcus dies in artificial cultures, frequent transfers on serum media must be made, and the cultures to be used for the immunizing injections must be freshly prepared each time in a plain agar medium. The question arises, therefore, whether the practical operations cannot be simplified by the employment, at least over certain periods, of killed cultures of the meningococcus so prepared as to prevent the autolysis which tends to destroy the antigenic properties. We have not carried out an exhaustive study of this subject but from many tests on rabbits and the following experiment with Horse M 28, we are of the opinion that the subject is worthy of thorough investigation.

Experiment 5.—Because of the rapidity with which the meningococcus autolyzes in cultures, growths in plain agar in Blake bottles, incubated at 37°C. for 8 to 10 hours, were employed. The surface growths were washed off with 20 cc. of isotonic saline solution and the heavy suspensions were quickly heated in a water bath to 65°C. to destroy the autolytic ferment. Test for viability was made and 0.35 per cent tricresol added. The suspension was kept in the refrigerator.

Horse M 28, after the usual preliminary treatment, was injected, beginning January 4, 1918, with suspensions of killed cultures of the same strains that were used in the immunization of Horses M 24 and M 25; *viz.*, No. 1 (para), No. 60 (regular), and Nos. 30, 31, and 38 (intermediates). The test serum bleedings were made after 7 and 12 months of immunization with the results shown in Table IV.

TABLE IV.

Number of Stock Strains Agglutinated by the Serum of Horse M 28 Immunized with Killed Cultures of Five Strains.

Duration of immunization.	Agglutination test positive.	
	1:400 or higher.	1:100
mos.		
7	46 of 60	59 of 60
12	53 " 56	56 " 56

This single experiment may be taken merely to indicate that as far as the agglutinin response is concerned, killed cultures of me-

ningococci can be used for immunizing horses. It will be patent from all the circumstances and from what has been stated above, that this fact is not regarded as tantamount to the conclusion that a therapeutically active and efficacious antimeningococcic serum can be produced in this way. We should, indeed, be willing to go no further at the present time than to propose that the suspended killed cultures be kept on hand to be used on occasion in place of the suspended live cultures when for any reason circumstances make it impossible to inject the latter. During the war when antimeningococcic serum was being produced at The Rockefeller Institute under great pressure, we never relied on the killed cultures alone and, at most, only occasionally alternated the injection of the killed and the living cultures in the routine manufacture of antimeningococcic serum. We still believe in the practice of employing live cultures and also of cultures freshly isolated from cases of epidemic meningitis.

The power of stored killed cultures, preserved with tricresol, to induce agglutinin formation was tested in one instance in rabbits. The killed cultures had been in the refrigerator for periods of 8 and 14 months. The rabbits tolerated the usual doses. One rabbit injected with a killed regular strain gave a serum of 1: 1,600 titer, and another injected with a killed para strain gave a titer of 1: 800 against freshly prepared killed cultures. The stored killed suspensions were agglutinated in other immune rabbit sera in high dilutions. As a conservative routine, however, the killed cultures employed occasionally for injection and for agglutination were not used after 3 months storage.

SUMMARY AND CONCLUSIONS.

In an attempt to simplify the manufacture of an efficacious antimeningococcus serum an experimental study has been made of a number of sera produced with a few or with single strains of meningococcus, the therapeutic polyvalent serum produced at The Rockefeller Institute with more than 50 strains being used as a standard of comparison.

It was found that horses injected with an antigen limited to five, three, or even one strain yielded sera with a range of agglutinins covering in high dilution practically all the stock strains used in pro-

ducing the polyvalent serum. These sera appeared to equal the polyvalent serum in range and titer of agglutinins, but on further examination fundamental differences were found. Storage for a year had little effect upon the titer and inclusiveness of the polyvalent serum, whereas the monovalent serum had fallen off greatly, especially in regard to secondary or subsidiary agglutinins, so that only a comparatively small number of stock strains was still agglutinated. The serum made with five strains, a regular, a para, and three intermediate meningococci, approached the polyvalent serum in keeping qualities and still agglutinated at the end of this period 39 of the 41 strains tested.

Absorption tests also brought out inherent differences in the nature of the polyvalent and the monovalent sera which had appeared to be practically identical in simple agglutination tests. The homologous strain on triple absorption was able to exhaust the monovalent serum completely, but was unable to remove from the polyvalent serum agglutinins to which 30 of 44 different strains were able to react. Absorption with another single strain of the same type removed from the monovalent serum agglutinins for a majority of the test strains but left the polyvalent serum relatively unaffected.

It is comparatively easy to produce a serum effective against about 80 per cent of the spinal strains of meningococci encountered. Deficiencies in our knowledge of the antigenic capacities of the meningococcus have led to the more or less empirical use of a large number of cultures in the preparation of a serum effective against the remaining 20 per cent of the strains. How far the number of the latter in the antigen may be reduced without restricting the efficacy of the serum remains yet to be determined. However, the experimental evidence recorded here apparently does not favor the use of an antigen limited to one or too few strains. For example, three or five selected strains produced a serum which agglutinated practically all the strains against which it was tested. But in view of the many observations which point to the greater therapeutic efficacy of a serum made with a larger number of strains we would not as yet advocate a serum prepared with too limited antigens even though it contains at first a wide range of agglutinins.

It has been brought out that a monovalent serum contains, in addition to specific agglutinins, a wide range of common or secondary agglutinins which tend to disappear during storage. The difference between specific and secondary agglutinins is not apparent in simple agglutination tests, but is revealed by absorption tests. It is probable that in a serum prepared with a few strains the same condition exists, whereas in a serum produced with a large number of strains the agglutinins are mainly specific as contrasted with the fact that most of them are secondary in the serum produced with few strains. The question whether secondary agglutinins are therapeutically equivalent to primary or specific agglutinins requires further study.

We wish to acknowledge our indebtedness to Dr. J. H. Brown and Dr. R. B. Little, of the Department of Animal Pathology of The Rockefeller Institute, for their cooperation.

INDEX TO AUTHORS.

INDEX TO SUBJECTS.

B

C

D

E

The Journal of
experimental medicine